

SPECIATION OF ZINC(II) IN MODEL SOLUTIONS
AND IN HUMAN SERUM BY ON-LINE DIALYSIS
AND VOLTAMMETRIC STRIPPING ANALYSIS

EDUARDO O. MARTINS



LUND 1984

1985



SPECIATION OF ZINC(II) IN MODEL SOLUTIONS AND IN HUMAN SERUM BY
ON-LINE DIALYSIS AND VOLTAMMETRIC STRIPPING ANALYSIS

by

Eduardo Martins, B.Sc., Ld

Akademisk avhandling som med vederbörligt tillstånd av matematisk-naturvetenskapliga fakulteten vid Universitetet i Lund för avläggande av filosofie doktorsexamen kommer att offentligen försvaras å Kemicentrum, Sölvegatan 39, sal F, fredagen den 18 januari 1985.

Avhandlingen försvaras på engelska.

Organization
LUND UNIVERSITY

Document name
DOCTORAL DISSERTATION

Date of issue
January 1985

CODEN:
LUNKDL/(NKAK-1010)/1-130 (1985)

Department of Analytical Chemistry

Author(s)

Sponsoring organization

Eduardo Martins

Title and subtitle

Speciation of Zinc(II) in Model Solutions and in Human Serum by On-line Dialysis and Voltammetric Stripping Analysis

Abstract

Analytical methods for the speciation of zinc in model and human sera were tested and developed.

Separation of the low molecular weight (LMW) fractions from other serum components was performed by flow dialysis and the determination of zinc bound to LMW ligands, like amino acids, by anodic stripping at a mercury electrode.

Modeling of flow-through analytical dialyzers and characterization of the permeation of ions and simple complexes through cellulose acetate membranes were studied.

The system, intended to be used in flow analysis, includes a flow-through dialyzer and a flow-through voltammetric cell, whose design and performance are described. The binding of metal ions to albumin was studied by ^{113}Cd NMR.

Key words

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language
English

ISSN and key title

ISBN

Recipient's notes

Number of pages 130

Price

Security classification

Distribution by (name and address)

Eduardo Martins, Dept of Analytical Chemistry,

University of Lund, P.O.Box 124, S-221 00 Lund, Sweden

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature

E. O. Martins

Date 1984-11-14

CONTENTS

	page
Abstract	I
List of papers	II
List of tables	III
1. INTRODUCTION	1
1.1 Biological	2
1.2 Clinical	2
1.3 Distribution	2
1.4 Intake and turnover	3
1.5 Transport	3
1.5.1 Circulating fractions in blood	3
1.6 Speciation of zinc in blood	8
2. PRESENT INVESTIGATION	11
2.1 Zinc binding to albumin: NMR studies	12
2.2 Separation: flow dialysis	14
2.2.1 Mass transfer	15
2.2.2 Permeability	17
2.2.3 Resistance to mass transfer	17
2.2.4 Sorption into the membrane	19
2.2.5 Diffusion in the membrane	21
2.2.6 Dialysis of complexes	22
2.2.7 Synopsis and conclusion	22
2.3 Detection: voltammetry	23
2.3.1 Anodic Stripping Voltammetry	23
2.3.2 Flow-through cell with mercury electrode	25
3. DIALYSIS OF MODEL AND HUMAN SERA	25
ACKNOWLEDGEMENT	27
REFERENCES	28

1. The first part of the report is devoted to a general description of the project and its objectives. It is followed by a detailed account of the methods used in the study, including the selection of subjects, the design of the experiment, and the procedures for data collection and analysis. The results of the study are then presented in a series of tables and figures, which are accompanied by a detailed discussion of their significance. Finally, the report concludes with a summary of the findings and a list of references.

2. The second part of the report is devoted to a detailed description of the results of the study. It begins with a summary of the main findings, which are then followed by a more detailed discussion of each of the results. This discussion includes a comparison of the results with those of previous studies, and an attempt to explain the results in terms of the underlying theory. The report concludes with a list of references.

3. The third part of the report is devoted to a detailed description of the methods used in the study. It begins with a summary of the main methods, which are then followed by a more detailed discussion of each of the methods. This discussion includes a comparison of the methods with those of previous studies, and an attempt to explain the results in terms of the underlying theory. The report concludes with a list of references.

4. The fourth part of the report is devoted to a detailed description of the subjects used in the study. It begins with a summary of the main findings, which are then followed by a more detailed discussion of each of the results. This discussion includes a comparison of the results with those of previous studies, and an attempt to explain the results in terms of the underlying theory. The report concludes with a list of references.

5. The fifth part of the report is devoted to a detailed description of the design of the experiment. It begins with a summary of the main findings, which are then followed by a more detailed discussion of each of the results. This discussion includes a comparison of the results with those of previous studies, and an attempt to explain the results in terms of the underlying theory. The report concludes with a list of references.

6. The sixth part of the report is devoted to a detailed description of the procedures for data collection and analysis. It begins with a summary of the main findings, which are then followed by a more detailed discussion of each of the results. This discussion includes a comparison of the results with those of previous studies, and an attempt to explain the results in terms of the underlying theory. The report concludes with a list of references.

7. The seventh part of the report is devoted to a detailed description of the results of the study. It begins with a summary of the main findings, which are then followed by a more detailed discussion of each of the results. This discussion includes a comparison of the results with those of previous studies, and an attempt to explain the results in terms of the underlying theory. The report concludes with a list of references.

8. The eighth part of the report is devoted to a detailed description of the methods used in the study. It begins with a summary of the main methods, which are then followed by a more detailed discussion of each of the methods. This discussion includes a comparison of the methods with those of previous studies, and an attempt to explain the results in terms of the underlying theory. The report concludes with a list of references.

9. The ninth part of the report is devoted to a detailed description of the subjects used in the study. It begins with a summary of the main findings, which are then followed by a more detailed discussion of each of the results. This discussion includes a comparison of the results with those of previous studies, and an attempt to explain the results in terms of the underlying theory. The report concludes with a list of references.

10. The tenth part of the report is devoted to a detailed description of the design of the experiment. It begins with a summary of the main findings, which are then followed by a more detailed discussion of each of the results. This discussion includes a comparison of the results with those of previous studies, and an attempt to explain the results in terms of the underlying theory. The report concludes with a list of references.

SPECIATION OF ZINC(II) IN MODEL SOLUTIONS AND IN HUMAN SERUM BY
ON-LINE DIALYSIS AND VOLTAMMETRIC STRIPPING ANALYSIS

This thesis is based on the following papers, referred to in the text
by their Roman numerals

- I Cadmium(II), Zinc(II) and Copper(II) Ions binding to Bovine Serum Albumin. A ^{113}Cd NMR study.
Eduardo O. Martins and Torbjörn Drakenberg
Inorg. Chim. Acta 67(1982)71-74
- II A Flow-through Cell for Differential Pulse Anodic Stripping Voltammetry
Eduardo O. Martins and Gillis Johansson
Anal. Chim. Acta 140(1982)29-37
- III Model Experiments for Determinations of Zinc-Amino Acid Complexes in Biological Fluids by Polarography
Eduardo O. Martins and Gillis Johansson
Anal. Chim. Acta 116(1980)53-60
- IV Solute Transfer in On-line Analytical Flow-through Dialyzers
Bo Bernhardsson*, Eduardo Martins and Gillis Johansson
*Dept of Mathematics
Anal. Chim. Acta, in press
- V On-line Dialysis of some Metal Ions and Metal Ion Complexes
Eduardo O. Martins, Mats Bengtsson and Gillis Johansson
Anal. Chim. Acta, in press
- VI Flow-through Dialysis of Zinc(II) from Model and Human Sera
Eduardo O. Martins

LIST OF TABLES

1. Example of functions performed by zinc compounds
2. Distribution of zinc in plasma
3. Scheme of the probable equilibria
4. Example of alteration in the distribution of zinc
5. Analytical methods employed in the speciation of zinc in blood

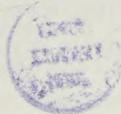
1. INTRODUCTION

1.1 Biological

Zinc is an essential element in biology. Its significance was first recognized in 1869 as a requirement for the growth of Aspergillus niger [1]. Although its occurrence in organic matter [2] and human liver [3] was reported in 1877, the importance of zinc as an essential nutrient in animals was not established until 1934 [4,5]. Evidence of zinc deficiency in man was first observed by Prasad et al. [6] and subsequently confirmed [7-10].

The biochemistry and metabolism of zinc have been abundantly reviewed [e.g. 11-14, 27]. Its presence and specificity in erythrocyte carbonic anhydrase, whose function in the transport of CO_2 in blood is well known, was established in 1940 by Keilin and Mann [15] and in the following decades almost 160 enzymes in which zinc is an essential part or an activator have been identified [16,17]. The ubiquitousness and special chemistry of zinc (a transition metal element with Lewis acid character, easy formation of low coordination number bonds, easy deformation of the coordination geometry and relatively rapid ligand exchange) have justified an impressive research effort in recent years and confirmed its importance on molecular and cellular levels.

The major functions appear to be enzymatic. Zinc has been associated in catalysis and inhibitory control [14], maintenance of structure of apoenzymes [22], regulation of various functions in cells [18], stabilization of macromolecules and biological membranes [19,20], synthesis of nucleic acids and control of cell multiplication [21-25], amino acids metabolism [26], regulation of intracellular cyclic-AMP and -GMP [28], membrane protection from radical oxidation [29], endocrine functions [30-34] and many other biochemical processes involving synthesis or degradation of proteins, lipids and carbohydrates [35-37].



1.2 Clinical

The use of zinc salts for medical purposes has been known since antiquity [38] but clinical interest did not arise until it had been proved that depletion of zinc in the blood was the cause of a rare type of dwarfism [6] and of a skin disorder, acrodermatitis enteropathica [39], both disfunctions reparable by zinc supplementation. Deficiency, depletion or change in the distribution of zinc have been found in numerous clinical situations [40-49]. Although it has not yet been definitely assessed for every case whether zinc is the decisive factor, its implication in infections [41,45], surgical trauma [46,47] myocardial infarction [48-55], alcoholic cirrhosis [55-60], alcohol withdrawal syndrome [61], total parenteral nutrition [62], hepatitis [63-64], pregnancy [65], impaired renal function [66-69], rheumatoid arthritis [70,71], acute viral hepatitis [72,73], wound healing [74], skin ulceration [75], burns [76] and tumors [11,77] is well documented. Toxicity due to excessive accumulation of zinc, apart from very few accidental cases, is nonexistent.

1.3 Distribution

The total amount of zinc in man is 1.4-2.3 g for the whole organism (2 g for an adult) [78]. It is found in all organs but in different concentrations and it is particularly abundant in specific tissues in the eye, prostate ($>7.7 \text{ } \mu\text{mol/g d.w.}$) and pancreas ($2.1\text{-}3.5 \text{ } \mu\text{mol/g d.w.}$). Intracellular zinc (the fourth most abundant metal within cells after K^+ , Ca^{2+} , and Mg^{2+} , $10\text{-}14 \text{ } \mu\text{g/g}$ for the erythrocyte), is distributed principally in the supernatant (53%), nuclei (20%), microsome (17%) and mitochondria (11%) [79].

Blood contains a small fraction of the total zinc (1.3%) distributed between plasma (12-22%), erythrocyte (75-88%) and leucocytes (3%). In terms of concentration, however, a single leucocyte contains 25 times as much zinc as a single erythrocyte.

1.4 Intake and turnover

The normal intake is 10-15 mg/day [80] and the average apparent absorption is about 50% [81,82]. The absorption pathway is similar to the other nutrients and probably regulated or controlled by small ligands and/or protein complexes in a relatively fast exchange [83], although the correct mechanisms are unknown. The turnover rate of injected zinc is 24 hours [84] although a minor fraction is retained for many weeks. Equilibria between zinc and the various biological compartments extend from rapid (a few hours for plasma and soft tissues), intermediate (muscle red cells and testis [85]) to extremely slow (bones and hair [86]). Turnover and accumulation are most rapid in the liver, spleen and kidney [87].

1.5 Transport

The mechanisms of transfer and transport of zinc from the absorption sites in the gastrointestinal tract to the liver and finally to the bloodstream are almost unknown. The experimental difficulties associated with the isolation of membranes without disrupting or upset intrinsic membrane proteins which can function as specific carriers makes the identification of the bioavailable species difficult [171-180]. A few LMW ligands assisting the transfer were, however, identified [88-93]. Transferrin has been suggested as one such candidate [94] in transferring zinc in portal blood but recent studies have indicated that albumin maybe a major carrier [95,96].

1.5.1 Circulating fractions in blood

In the liver [97] zinc is transferred to the circulating proteins in the blood. Two pools, based on exchange rates, have been identified [40,98,99]. In the non-exchangeable fraction, α_2 -macroglobulin, α_2 -M, which incorporates ca 30% of the plasma zinc (total 1 mg l^{-1}) is the main ligand [99]. The exchangeable fraction consists principally of albumin [58,100] and amino acids [100,101]. In recent studies other plasma proteins were also indicated as potential zinc ligands [102-106] but the protein-bound fraction associated with

others than albumin or α_2 -M is of minor quantitative importance (<3%), [103]. A specific transport protein for zinc in the plasma, has, however, not yet been identified.

Albumin is the principal transport protein in the blood [108,109] with a high binding capacity for metals, hydrophobic ligands and drugs [see e.g. 113-115]. It is also the most abundant protein in the human plasma (60% of the total, concentration 40 g L^{-1} , $\sim 0.6 \text{ mM}$, [110]) and its amino acid sequence has been determined [111,112]. The interaction between human serum albumin, HSA, (or its model, bovine serum albumin, BSA [116]) and zinc ions has been extensively studied and a wide range for the binding parameters, depending on the analytical methodology, have been reported. For the high affinity binding site involving two or probably three histidinyll residues (which seems to be the only with physiological relevance, because of the large excess of albumin present in normal conditions) an apparent binding constant of 10^7 M^{-1} is proposed [100].

The presence of free amino acids, FAA, in human blood plasma was first established by van Slyke and Meyer [117]. The concentration of amino acids in healthy subjects extends from a few μM (aspartic acid) to 0.56 mM (glutamine). Amino acids are easily complexed with metals and at physiological pH zinc ions are coordinated through α -amino or α -carboxyl groups, with stability constants of ca $10^{4.5}$ - $10^{5.5} \text{ M}^{-1}$, with exception of histidine ($10^{6.5} \text{ M}^{-1}$) and cysteine (10^9 M^{-1}). The total concentration of FAA is ca 1.9 mM . Amino acids are able to compete with plasma albumin for zinc ions in vitro [100,101] or in vivo [118-120] and it has been shown that cysteine and histidine are the only important amino acid ligands for zinc.

Mixed ligand complexes, MLC, are suggested to be present in biological fluids, in particular blood, and their importance in controlling the distribution of metals amongst LMW ligands has been the subject of numerous investigations [e.g. 121,122]. They are present in complicate systems of labile equilibria at extremely low concentrations, very difficult to quantify by common analytical methods, and only in the case of copper, MLC have been isolated [135]. Oligopeptides are, for the same reasons, difficult to isolate and charac-

terize and artificial methods, e.g. computer simulation, have been widely used to predict multiple equilibria distribution. Interaction between all possible LMW ligands, amino acids or small peptides, is a huge analytical and methodological task (for di-, tri- and tetra-peptides the number of interactions concerned amount to 400-8000-160000, respectively) and consequently very few studies have appeared to date. MLC functions appear to be associated with storage and transport of active compounds through membranes. Mixed complexes formed between zinc and cysteine and/or histidine are thermodynamically favorable i.e. their stabilities are enhanced in relation to simple complexes [123,124].

Human alpha-2-macroglobulin is the principal ligand in the non-exchangeable (slow exchange) fraction and accounts for ca 30% of the bound zinc in plasma [99,125]. It is a glycoprotein of MW 718000 [126] and it was first isolated by Schultze et al. [127]. The amino acid and carbohydrate composition of the α_2 -M have been determined [126,128-130,133]. Its precise function as a zinc metallo-protein is still undefined but it has been shown to play a significant role in the elimination and inhibition of endopeptidases [131]. The concentration in plasma is 130-334 mg/100 ml [132] and isolated α_2 -M contains 80-100 μ g of zinc/g of protein [134]. Two classes of binding sites and an apparent binding constant for the high affinity site of $3.06 \cdot 10^7 \text{ M}^{-1}$ has been reported [131].

Besides HSA and α_2 -M, eleven other circulating proteins are able to bind zinc in serum (i.e. radioactive zinc is present in all separable protein fractions). Transferrin (serum concentration 23-36 μ M), ceruloplasmin (0.17-0.24), immunoglobulins, G, A, M (1.2-1.8), haptoglobin (3-19), prealbumin (4.6-5.7) and α_1 -antitrypsin (46-111) share the remaining 2-3% of the protein-bound zinc [101-105] but their relative importance in the quantification in the transport of zinc in plasma is minimal. The function of the individual fractions with exception for a few well characterized compounds (Table 1) is, however, in large grade, unknown.

TABLE 1. Example of functions performed by zinc compounds

SPECIES	FUNCTION OF THE ZINC ION	FUNCTION OF THE COMPOUND	SYSTEM	AFFINITY a)
Zn: Metalloenzymes (Zn activated enzymes)	Attack of enzyme bound CO ₂ by ZnOH	Hydration of CO ₂	Carbonic anhydrase, erythrocyte	>10 ¹⁰
	Binding of PO ₄	Hydrolysis of phosphate monoesters	Alkaline phosphatase, erythrocyte	10 ^{5.6}
Zn: Metalloproteins	Structural	Binding and elimination of proteinases	Zn-α ₂ -Macroglobulin, serum	3·10 ⁷
Zn: Proteins	Unknown	Carrier	Zn-Albumin, serum	10 ⁷
Zn: Proteins	"	Transport and homeostasis of metals	Zn-Histone rich glucoprotein, serum	>10 ⁷
Zn: MMW Proteins	"	Regulation of zinc metabolism	Zn: Metallothionein, liver cyto- sol	>10 ⁷
Zn: LMW Ligands	"	Transport b)	Zn: Amino acids, serum	>10 ^{4.5}

a) Operationally defined as the association constant

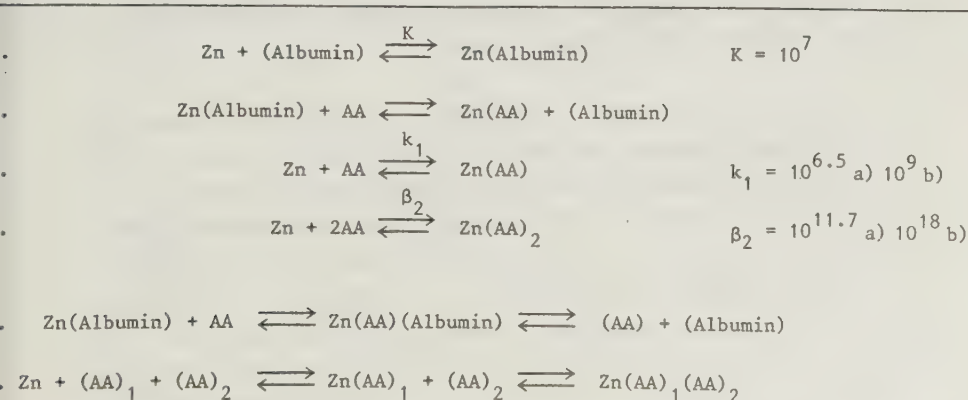
b) Assisting the transfer through membranes, mechanism unknown

The distribution of zinc amongst the components of plasma is summarized in Table 2 and the most relevant equilibria are schematically shown in Table 3.

TABLE 2. Distribution of zinc in plasma

FRACTION	SPECIES	CONCENTRATION	
		%	μM
Non-exchangeable (slow-exchange)	A. Metalloprotein complex: Zn- α_2 Macroglobulin	30-33	5
Loosely-bound (in labile equilibria with other ions in solution)	B. Albumin bound	60-65	10
	C. Amino acid bound	1-2	0.03
	D. Carboxylate Carbonate Phosphate Citrate Salysilate	} <0.1	$10^{-6} - 10^{-7}$ (estimated)
	E. Probably ZnCl_2		
"Free"		~ 0	$10^{-7} - 10^{-8}$ (estimated)
Probably in fast exchange	F. Bound to other circulating proteins	$\sim 2-3$	0.03

TABLE 3. Scheme of probable equilibria



a) Histidine

b) Cysteine, AA: amino acids

In blood zinc exists almost exclusively associated with proteins. Alteration in their relative distribution may be the cause or is related to a particular disfunction. A systematization is, at the present, forcibly defficient but as Table 4 shows the need of a more elaborate characterization of the different fractions is apparent.

1.6 Speciation of zinc in blood

Speciation of an element is defined as the identification of the chemical forms of the element in a specific phase, sample or compartment. In biological systems, the distribution of zinc can be characterized within three main groups:

1. The metal is strongly bound, with large binding constants, as in enzymes or in covalent bonds and for which the separation method does not dissociate the metal from the ligand.
2. The metal is bound to small molecular compounds, like amino acids, with defined binding constants and ligand concentrations and the distribution of species can be calculated from equilibrium equations.
3. The metal is bound to a large number of different ligands, as in blood, with varying ligand concentracions, usually not defined, and for which the separation methods can disturb the equilibrium between metal and ligands.

The first group is, perhaps, the easiest to speciate and characterize by well proven separation methods, like equilibrium dialysis, gel filtration and chromatographies. For the second group, speciation can, usually, be based on model studies and the determination of free metal ion by methods such as ion selective electrodes, and voltammetry, which, however, lack sensitivity and selectivity. For the third group, no straightforward methodology is, at the present, available. The characterization of the different fractions by methods as enzymatic assays, or ion-exchange, involve the disruption of equilibria might it be as a result or the adjustment of pH, interaction with buffers or adsorption effects.

TABLE 4. Examples of alteration in the distribution of zinc

TOTAL ZINC	PROTEIN BOUND ZINC		URINARY ZINC	PROTEIN		OTHER	CLINICAL SITUATION	REF.
	HSA	OTHER		TOTAL	HSA			
Decrease	Decrease						Alcohol withdrawal	61
Decrease	Decrease		Increase				Histidine admini- stration in sclerosis	181
Unchanged	Unchanged		Increase	Unchanged		Urinary AA unchanged	TPN	62
Decrease	Decrease	Unchanged α_2 -M-Zn increase of γ -globu- lin bound zinc		Decrease	Decrease	Increase of γ -globulin	Hepatic cirrhosis	58
Decrease	Decrease	Unchanged α_2 -M-Zn		Decrease	Decrease	Unchanged α_2 -M increase ceru- loplasmin	Pregnancy	65
Decrease	Decrease			Decrease	Decrease	Increase of α_2 -M	Myocardial infarc- tion	48, 210
Decrease			Increase				Malignant melanoma	182
Unchanged	Unchanged		Increase	Decrease	Unchanged		Renal failure	66
Decrease	Decrease			Unchanged			Neurological di- seases	183
Decrease	Decrease		Increase				Alcohol cirrhosis	184
Decrease	Decrease		Unchanged				Biliary cirrhosis	184
Decrease	Decrease		Increase				Renal transplants	185

In plasma (or blood) the strategy has been on separation of the proteic fraction and subsequent determination of the metal in these fractions. Table 5 summarizes the most common techniques in the speciation of zinc in plasma.

TABLE 5. Analytical methods employed in the specification of zinc in the blood

SEPARATION/FRACTIONATION	DETECTION	REFERENCES
Ultrafiltration	AAS, ^{65}Zn liquid scintilation	136
Equilibrium dialysis	AAS	138
Equilibrium dialysis-immuno-adsorbent chromatography	^{65}Zn liquid scintilation	107
Equilibrium dialysis - electrophoresis	AAS	101
Ultracentrifugation	AAS	140
Sucrose density-gradient centrifugation	AAS	134
Salt fractionation	AAS	137
Polyethylene glycol precipitation	AAS	125
Electrophoresis	AAS	58
2D-immunoelectrophoresis	^{65}Zn liquid scintilation	102
Chelating ion-exchange	AAS	141
Affinity chromatography	AAS	103
Gradient chromatography	AAS	138
Gel filtration	AAS, ^{65}Zn liquid scintilation	105, 140, 96
Gel filtration - ion-exchange chromatography	AAS	99, 139
Gel filtration - ion-exchange chromatography - electrophoresis	^{65}Zn liquid scintilation	94

The measurement has usually been performed by instrumental methods. Atomic absorption spectroscopy, AAS, which displays very favourable characteristics with regard to sensitivity, precision, selectivity and determination rate, with its various forms of flame and flameless atomic emission or absorption spectroscopies, has found widespread application [142,160-164]. Direct determination after convenient dilution of the analyte or determination followed the destruction of the protein matrix by digestion or extraction have been used.

Other detection methods include neutron activation analysis [143,165], X-ray fluorescence spectrometry [144], emission spectrometry [145], atomic fluorescence [146], proton induced X-ray emission spectrometry [166], spectrophotometry [167,168] and anodic stripping voltammetry [147,169,170].

2. PRESENT INVESTIGATION

The present thesis is part of a project aimed at the development and application of analytical methods for the speciation of zinc in biofluids. Within the project the detection of zinc ions with ISE and with enzyme immobilized reactors have already been reported [148,149].

This thesis describes the combination of flow dialysis and electrochemical detection for the determination of the dialyzable fraction of zinc from model serum. In Paper I the binding of metal ions to serum albumin was studied by NMR techniques. The design and the performance of a flow-through voltammetric cell with a mercury electrode are presented in Paper II. Paper III reports on the studies of the transport of zinc amino acid complexes through hollow-fiber dialyzers and their detection by differential pulse polarography. Papers IV and V have a twofold intention: to model and to study the performance of parallel plate flow-through dialyzers and to contribute to the understanding of the transport of ions and simple complexes through cellulose acetate membranes. Finally Paper VI studies the measurement with anodic stripping voltammetry of dialyzable zinc from model and human sera.

2.1 Zinc binding to Albumin: NMR studies

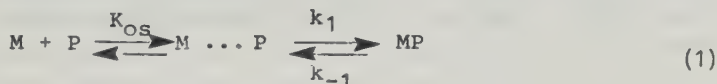
Nuclear magnetic resonance, NMR, is a spectroscopic technique based on the fact that atomic nuclei oriented by a strong magnetic field absorb radiation at characteristic frequencies [150,151]. Although NMR is inherently a insensitive technique it is one of the most powerful tools to study ion binding at the molecular level. Information on molecular structure, conformation, molecular motion and other rate processes can be extracted from the analysis of the spectral lines [152]. Particularly important is the fact that the nuclei of the same element in different chemical environments resonate at distinct frequencies, i.e., they are chemically shifted [153]. The parameters measured on NMR (chemical shifts, coupling constants and relaxation rates) are averages over a population of nuclei and over time. The physical event defines a time scale and all the molecular events occurring in this time scale will be reflected on the measurement. In NMR the fundamental time scale is defined by a nuclear precession frequency. Chemical shifts, ν , are defined in relation to the frequency of the free (aquated) ion. Transversal or spin-spin relaxation time, T_2 , is the time constant for the decay of the magnetization on the xy-plane and for spin-spin relaxation described by a single exponential (as for $I=1/2$ nuclei), T_2 (and R_2 , relaxation rate) is directly measurable from the signal as half-height frequency, $\Delta\nu_{1/2} = (\pi T_2)^{-1}$.

Depending on the nature of the exchange process the different environments between which the exchange is occurring will be characterized by different values of the NMR parameters. Three criteria are usually followed: slow, if the exchange rate $k \ll |\nu_A - \nu_B|$, ($k \ll (|T_A^{-1} - T_B^{-1}|)^{-1}$), intermediate if $k \approx |\nu_A - \nu_B|$, and fast if $k \gg |\nu_A - \nu_B|$, where ν_A and ν_B characterize the chemical shifts in two environments A and B. In terms of lifetimes $\tau (=k^{-1})$ of the individual states the inequalities are reversed.

For small ions in solutions of macromolecules there is an exchange between the bulk solution and one or more classes of binding sites on the macromolecule. For slow exchange conditions the intensities of

NMR signals can be used to determine binding constants and stoichiometries. When the exchange is rapid compared to the relaxation of bound ions the observed rate is given by $R_{\text{obs}} = P_b R_b + P_f R_f$, where P_b is the fraction of ions bound to the macromolecule and R_b and R_f the relaxation rates of bound and free ions, respectively.

In the formation of a cation-protein complex the following mechanism applies [154]:



K_{os} is the equilibrium constant for the formation of the outer-sphere complex and k_1 and k_{-1} are the first-order rate constants for the formation and dissociation of the final complex. The overall rate constant for cation binding is $k_{\text{on}} = K_{\text{os}} k_1$ and the dissociation rate $k_{\text{off}} = k_{-1}$ is given by $k_{\text{off}} = k_{\text{on}} / K_B$, where K_B is the equilibrium constant of cation binding.

Not all elements are amenable to NMR. Zinc is silent in various spectroscopies but can easily be replaced by spectroscopically active probes like ^{111}Cd or ^{113}Cd , both spin one half (in contrast to the quadrupolar ^{67}Zn which gives rise to extra broadening) and are very sensitive in terms of chemical shift range [155,156]. ^{113}Cd has been substituted for zinc in the study of zinc proteins [157,158].

^{113}Cd -NMR and the principles outlined above were employed in the study of the interaction of Cd, Zn and Cu to serum albumin [Paper I]. Two classes of binding sites were identified: two sites with high affinity binding ($K > 10^7 \text{ M}^{-1}$) and a number of weaker sites ($K 10^4 \text{ M}^{-1}$).

Competition between potential ligands (L-histidine, L-lysine, L-cysteine, β -glycerophosphate, glycerodiphosphate, glucosamine, glutamine, palmitic acid, stearic acid, cholesterol, bilirubin and transferrin) and albumin for zinc ions, under the experimental

conditions as in Paper I, and at ligand concentrations up to six times of those of albumin revealed that no ligand was able to displace zinc ions from the specific Cu, Zn high affinity site [159].

2.2 Separation: flow dialysis

Separation of chemical species based on the difference of their transport rates under the action of one or more driving forces through synthetic membranes has found widespread application in numerous fields as desalination, food and drugs industries, artificial kidney, fractionation of macromolecular solutions, removal of effluents and many others. Transfer of mass through a membrane may be caused by diffusion of the individual molecules or by convective flow induced by an electric field, or a concentration, pressure or temperature gradient. Transport rates are determined by the forces acting on the individual components and their concentration and mobility within the membrane. Depending on the nature of the forces involved, the processes have different designations. When only concentration gradient is the driving force for the transport of mass through the membrane, the process is called dialysis. The transport is a non-equilibrium process and the same phenomenological equations relating the flow of matter to the corresponding driving force apply formally to every process. Rigorously, the transport of chemical species through a membrane is proportional to the gradient in the chemical potential, which consists of additive terms of the gradients in hydrostatic pressure, in the concentration and in the electric potential [190]. Practically, however, the transport of mass is defined by a phenomenological permeability which is in turn defined in terms of concentration or pressure outside the membrane and measurable experimentally.

Dialysis, the first membrane separation process to be discovered [186,187] has since been used almost exclusively in preparative biochemistry as a simple tool in protein isolation and purification. By its nature a relatively slow method (transport rates are slow and the concentration gradients are usually set by the system) and not

very selective it has nevertheless found an important application in the medical field with hemodialysis [188].

The development of dialysis has long been conditioned by the development of membrane technology [189]. The appearance of thin-film polymer membranes like cellulose acetate, with uniform porosities and stable properties or hollow-fibers [191] with large surface to volume ratio much contributed to the acceptance of dialysis as a reliable and reproducible separation procedure in analytical chemistry.

The studies of Craig [192], Steward [193] and Dean [194] set the basis of modern dialysis. Flow dialysis, either in the thin film or hollow fiber configuration has been used in binding studies [195-198] or as components in flow analysis [199-201,202].

2.2.1 Mass transfer

A flow through thin film dialysis system can generally be described as a membrane separating two solutions flowing in channels, laminae or tubing, one of which carries the substance (ions or molecules) to be transferred to a receiver solution flowing in the opposite side of the membrane. Common to all transfer processes to interphases, the mass transfer in such a system consists of the following stepwise processes:

- A. Convective transport to the boundary layer
- B. Diffusion through the boundary layer
- C. Sorption into the membrane
- D. Diffusion through the membrane
- E. Desorption out the membrane
- F. Diffusion through the boundary layer
- G. Convective transport out of the boundary layer

In its simplest form the flux of a solute through a membrane J_i can be described by Fick's law

$$J_i = A j_i = -AD(\partial c_i / \partial z) \quad (2)$$

where A is the area across which diffusion occurs, j_i the flux per unit area, c_i the concentration and z the distance. In flow dialysis a new situation is, however, set forth; diffusion coefficients have to be connected to fluid flow arising from forced convection. Among the theories describing interfacial mass transfer (thin film, penetration, surface-renewal, boundary layer and Gratz-Nusselt, (GN), problem) [203], the later is very adequate to represent the new situation. For steady state, laminar, Newtonian and hydrodynamically fully developed flow, conservation of mass results:

$$\bar{v}(y) (\partial c / \partial x) - D (\partial^2 c / \partial y^2) = 0 \quad (3)$$

where x is in the direction of flow. The solution of eq. 3 is of course dependent on the boundary conditions set for the system [204], and for the GN problem with parabolic flow ($\bar{v}(y) = (1 - a^2/x^2)$) the mass transfer to the membrane is given by:

$$Sh = \text{const.} (Re)^{1/3} (Sch)^{1/3} (L/R)^{1/3} \quad (4)$$

Sh , Re and Sch are dimensionless variables, defined:

$$Sh = \text{Sherwood number} = K l / D$$

$$Re = \text{Reynolds number} = L \bar{v} / \nu$$

$$Sch = \text{Schmidt number} = \nu / D$$

K , mass transfer coefficient, D diffusion coefficient, l membrane thickness, L length, $\bar{v}$ average velocity and ν kinematic viscosity. Expressed in forces and fluxes, $Sh = (\text{mass transfer velocity})/(\text{diffusion velocity})$; $Re = (\text{inertial forces})/(\text{viscous forces})$ and $Sch = (\text{diffusion of momentum})/(\text{diffusion of mass})$.

Eq.4 describes the transfer from one channel. To fully describe transfer of mass from the sample to the receiver channel the solutions of the two convection-diffusion equations have to be coupled via a continuity of flux boundary condition at the membrane surfaces. This problem, a conjugate boundary value problem, has been solved for a variety of geometries in hemodialyzers [205-209] and adapted to analytical flow-through dialyzers in Paper IV. Two models

and the respective numerical solutions were set for plug and parabolic flows in both channels, and compared to a mixed-cup model originally derived by van der Linden [200] for gas diffusion.

For flow-through dialyzers with equal channel heights and for equal flow rates in both channels, a simplified expression can be derived from eq.17, Paper IV. The normalized output concentration is then

$$C = 1/2[1 - \exp(-2pL/av)] \quad (5)$$

where L is the length, p , permeability, a channel height and v flow rate.

2.2.2 Permeability

The transport of fluids across membranes can be characterized by a fundamental relationship:

$$\text{Permeability} = \text{Solubility} \times \text{Diffusivity}$$

Permeability coefficients of different units can be derived from flux data by applying various hypothetical membrane structures and/or driving forces [211-222] regardless of the actual and true mechanism of the process. In dialysis the diffusive permeability is simply defined from Fick's law:

$$\text{Permeability} = (DK/l) = \Delta C/j_i \quad (6)$$

and it is expressed in $(\text{length}) \times (\text{time})^{-1}$ units. In chemical engineering permeability is $(\text{Sh})^{-1}$.

2.2.3 Resistance to mass transfer

The efficiency of mass transfer process depends on the minimization of the resistances to mass transfer in each step. Each interface has associated a stagnant boundary layer with thickness varying with the hydrodynamic and diffusion parameters, through which the movement of

the solute is diffusive. In many cases the magnitude of this diffusional boundary layer resistance may be comparable or even greater than the intrinsic resistance of the membrane itself [217]. The total or overall permeability is a measure of the permeation through the membrane and the two boundary layers in each side. The total resistance to transport, R , is the sum of the intrinsic membrane resistance, R_m , and boundary layers resistance R_b :

$$R = R_m + 2 R_b \quad (7)$$

For stirred diffusion cells, batch of flow dialyzers, R is defined as

$$R = (C_1 - C_2)/J \quad (8)$$

where C_1 and C_2 are concentrations of the solute in either side of the membrane and J the solute flux. Only when $R_m \gg R_b$ will measurements of solute transport yield information on R_m . R_m is not directly accessible to experimental determination. R_b can, however, be analyzed and separated from the total permeability. There are usually two expedient approaches to determine the intrinsic transfer coefficient or true permeability, both based on the extrapolation of an experimental parameter and therefore both inaccurate. The flux of mass is determined for various thicknesses of the membrane [223] or for various stirrer speeds or fluid velocities [224-230]. Extrapolation of the membrane thickness or velocity (raised to a power to get the best fit of the data) to infinite will give R_m . The second approach was adapted in determining the permeabilities in Papers IV and V. There are, however, two sources of errors in the later approach:

1. Mass transport data are normally represented as

$$Sh = Re^c \cdot Sch^b \quad (9)$$

with c , b , empirical coefficients.

Although $b = 1/3$ agrees well with boundary layer theory [231] and experimental evidence, $c \sim 2/3$ depends on the geometry of the

experimental arrangement: large errors are incurred by extrapolating the data to v^a or $\omega^a = 0$. The value of the intercept is very sensitive to slight changes in the value of R .

2. For high Re numbers there is a laminar-to-turbulent type of transition in the mass transfer resistance. Since the data are generally obtained at limited range, extrapolation to high velocity rates will not give accurate results.

2.2.4 Sorption into the membrane

Sorption of ions to the membrane can conveniently be depicted by the partition between the aqueous and the membrane phases. Experimentally it can be obtained by the sorption-desorption method [222,232], i.e., measuring the time dependent concentration of a solute in an aqueous phase in contact with the membrane, the membrane removed from the solution and placed in a solute-free desorbing medium in which the solute concentration is again followed with the time. Long equilibration times are required to ensure that equilibrium is achieved. Partition coefficients in Paper V were obtained by this method.

The distribution of inorganic solutes between the aqueous phase and the cellulose acetate membrane is governed by four interrelated factors [232-233]:

1. Concentration of fixed charges in the membrane
2. Dielectric exclusion
3. Ionic association
4. Swelling and shrinkage of the membrane

The relative importance of the factors above mentioned is conditioned by the nature, concentration and charge of the permeating solute. Fixed charges in the membrane arise from the presence of carboxyl groups which exist in weak acid equilibrium and thus depend on the the pH of the medium. Cellulose acetate has an isoelectric point at ca pH 3 and at pH>3 there is an excess of negative charges and a preferential adsorption of cations. Charges cannot, however, exist isolated, they are neutralized by counter-ions and thus ion-exchange

mechanism between the permeating ions and the counter-ions in the membrane is thus probable.

The dependence of the partition coefficient on the dielectric constant of the medium is given by expressions of the form:

$$\ln(c_m/c_w) = [(-e^2 |Z_c Z_a|)/(2bkT)] (1/\epsilon_m - 1/\epsilon_w) \times \text{const} \quad (10)$$

where e is the electrostatic charge, $Z_c Z_a$ product of the valence of the ions, ϵ_m and ϵ_w , dielectric constants of the membrane and aqueous solutions, b mean ionic radius. ϵ_m refers to the whole membrane phase and therefore dependent on the water content of the membrane. The influence of the dielectric exclusion of charged solutes can be the dominant factor when ionic concentrations in the membrane greatly exceed the concentration of fixed charges. Eq.10 predicts the variation of partition with ionic size and charge, which has been experimentally verified [232,234, Paper V].

The third factor, ion-pairing, although reducing the effects of dielectric exclusion and fixed charges in the membrane, is normally insignificant at the low concentrations (< mM) dealt with in flow dialysis. Swelling and shrinkage of the membrane, effects of salts present in the polymer network, like CuSO_4 and ZnCl_2 [238] employed in the membrane manufacture, alter permeabilities but their effects are difficult to separate or predict.

For binary salt solutions, phase-equilibrium studies are scarce. Theory predicts an ion-displacement or common ion effect in explaining the decreased sorption of impermeable ions upon addition of a more permeable ion [233,235]. Experimental evidence in Paper V partly supports ion displacement mechanism.

Composition, ionic strength and pH of the solutions may play a non negligible role in the dialysis rate, as already suggested by Craig and King [239] and further confirmed in Paper V.

2.2.5 Diffusion in the membrane

The transport of solutes in the membrane is a diffusional process and as such governed by the same physical laws as all diffusion processes. Of importance to dialysis is the dependence of the diffusion coefficient upon molecular volume, temperature and viscosity, as reflected by the Einstein-Stokes law:

$$D = RT/N 6 \pi r \eta \quad (11)$$

r is the Stokes law radius and η the viscosity of the solvent.

Dependence of D on concentration, as given by [236]:

$$D = -nRT(1 + C d \ln \gamma / d \ln C) \quad (12)$$

or multicomponent diffusion, e.g. [237], although contributing, certainly, to the transport and because the variation in partition coefficients are many orders of magnitude larger than the variation of D , result that differences in diffusion tend to be much less important than differences in solubilities. In Paper V, however, this assumption was not fully verified and though the experimental data are, of course, insufficient, a membrane model with two different phases [234] is proposed.

Traditionally, equilibrium dialysis has been explained with help of two simplified models: a solution-diffusion model which accounts for electrostatic effects between permeates and membrane, and a pore model which views a membrane as a heterogeneous phase with pores and channels. The general equation of dialysis, which reads:

$$dc/dt = (-RT/N 6 \pi) (A_{\text{eff}}/nr) \text{grad. } c \quad (13)$$

where A_{eff} is the effective area for diffusion of a spherical molecule through a perfect cylinder, does not account for interactions within the membrane, which are found to exist. For organic and uncharged solutes, the permeation of a chemical species relative to another species largely depends on the ratio of their molecular sizes and roughly a function of the square root of their molecular

weight. For charged solutes, the true representation of flow dialysis of dilute solution, will involve, however, contributions from both models. A general equation which can predict, given the membrane characteristics (polymer composition, water content, porosity and thickness) the permeability of a given solute (charged or uncharged) is not available.

2.2.6 Dialysis of complexes

Dialysis of zinc amino acid complexes was studied in Papers III and V. In Paper III a cellulose acetate hollow fiber dialyzer was used so the results are not directly comparable with those obtained in parallel-plate flow-through dialyzer. Although the rate of dialysis for zinc amino acid complexes or zinc aminopolycarboxylic acids decreased compared to those of simple salts as expected, in accordance to the differences in diffusion coefficients, no straight-forward correlation could be found with the charges of the individual complexes. The differences in charges and protonization were found to have little effect on the transport rate, in accordance with Craig and Ansevin [240].

2.2.7 Synopsis and conclusion

There are a number of phenomena and parameters to consider in flow-through dialysis, from geometry to membranes. No single mechanism can model the transport of very different chemical species, ions and molecules, through membranes. Despite dialysis is not yet fully apprehended in all its details, it is, nevertheless a very convenient, reproducible and a broad application separation method.

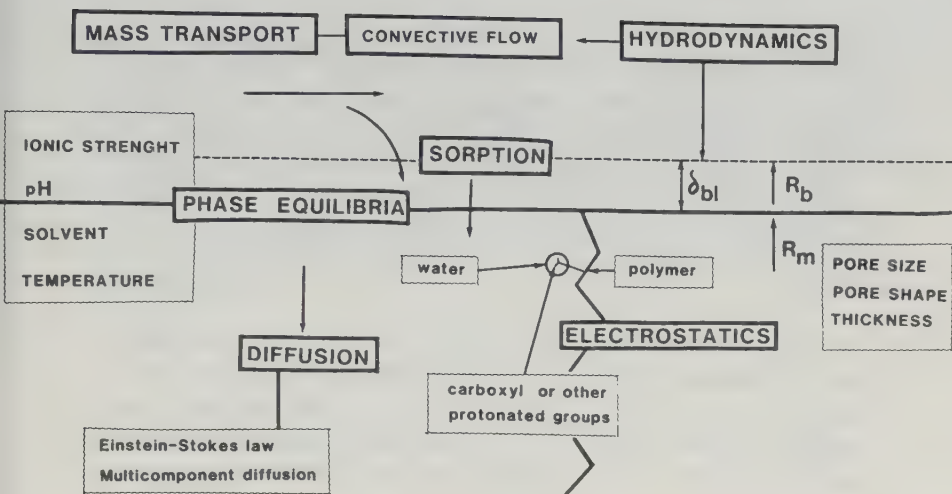
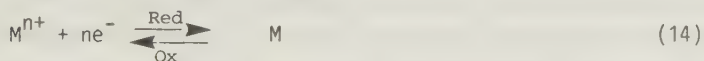


Fig. 1. Schematic representation of the mechanisms involved in flow dialysis

2.3 Detection: voltammetry

2.3.1 Anodic Stripping Voltammetry

Anodic stripping voltammetry, ASV, [241,242] is essentially an electrolysis at a microelectrode, leading to the reduction of a metal M^{n+} in the electrode and the subsequent oxidation to its ionic state according to the general equation:



The adjusted parameter is the potential E , the measured response is the resulting current i and the corresponding current-potential curve is the recorded voltammogram in which the peak-height is proportional to the bulk concentration of the respective metal dissolved as an ionic species in solution. An important property in voltammetry which explains its high sensitivity is the fact that one mole undergoing a redox process at the electrode generates a high electrical charge of 96500 n Coulombs.

The complete procedure in ASV involves a deposition step with the electrode at a sufficiently negative potential to reduce the metal, and with forced convection of the solution to the electrode, a short rest period and a potential scan in the anodic direction. At the negative electrode potential values an aliquot of the respective metal amalgam is reoxidized according to eq.14 and the current recorded.

In every redox reaction at an electrode there is always associated with the Faradaic process a capacitive component resulting from double layer charging. The measured current is thus the sum of the two contributions, one of which i_c , does not contribute to the process and limits the sensitivity of the method. To reduce the contribution of i_c to the signal, pulse methods are employed. In the differential pulse mode, DPASV, the potential is scanned in the form of a train of small rectangular voltage pulses from the deposition potential. The current is measured only during two time intervals situated just before each pulse and in the last phase of its duration. After each pulse, the Faraday current, i_F , decays with $t^{-1/2}$ but the charging current decays exponentially. In the final phase of each pulse, when the current is measured, i_c has decayed to negligible amounts and consequently, $\Delta i \approx \Delta i_F$. In this manner only part of the current flowing during each pulse duration is recorded and a very good signal-to-noise ratio is achieved.

The species detected in ASV, designated electroactive species, is not necessarily restricted to the aquo ion. For electrochemically reversible systems, however, the species being reduced cannot be distinguished [243,244]. In systems containing metal and ligands a set of mobile equilibria may exist between the various complexes and the aquo ion. If the rates of exchange of the various equilibria are much faster than the time scale in which the measurement is performed the current will be almost the same as that of the simple aqueous ions, allowance made for differences in diffusion coefficients, but the reduction potential will be shifted to more negative values.

2.3.2 Flow-through cell with HMDE

The efficiency in ASV is conditioned by the rate at which the metals arrive at the electrode surface, the diffusion properties of the electrolyte solution, the area of electrode and the time of the reduction, or plating, step. For a single metal ion being reduced at an electrode surface the current flow can be approximated by the Levich equation:

$$i(t)_{\text{dep}} = \text{const.} \times nFA D^{2/3} v^{1/2} v^{-1/6} c(t) \quad (15)$$

where A is the electrode surface area, v the velocity or stirring rate, ν the kinematic viscosity and c(t) the ion concentration at deposition time t.

The classic polarographic cell in which the transfer of mass is achieved by stirring the solution has been substituted, with apparent advantages, by flow-through cells, one of which, adapted to a hanging drop mercury electrode, HMDE, is described in Paper II. Cells of this type, with convenient geometries and small volumes, can be integrated in closed flow systems (Paper VI) which facilitates degassing, and matrix exchange techniques, decreases contamination risks and easily automated.

3. DIALYSIS OF MODEL AND HUMAN SERA

A closed system including a flow-through dialyzer and a voltammetric cell was set up in the determination of the dialyzable zinc (Paper VI). The dialysis of zinc from various types of synthetic serum and human serum revealed that the transfer of zinc was faster from human serum which may indicate the presence of dialyzable zinc complexes (MW < 1000-2000 Daltons). The experimental difficulties in analyzing zinc at these low concentrations, nearly at the detection limit of DPASV (1 nM), and the absence of convenient analytical methods preclude, however, any attempt to separate and identify the dialyzable species. The merits of the approach as a complement in the

speciation of zinc in biological fluids in a routine basis are, however, apparent: relatively inexpensive analytical instrumentation, easily automation and acceptable determination rate and a possibility of rapid screening of the distribution of zinc and other metals, particularly of those with toxicological interest.

ACKNOWLEDGEMENTS

I express my gratitude to Professor Gillis Johansson for his interest and continuous support during the course of this work. My thanks are also due to Torbjörn Drakenberg for his pleasant collaboration and competence and to Annica Bergkvist and my colleagues, too many to be mentioned, who contributed to an agreeable and fruitful stay at the Department of Analytical Chemistry.

Finally, my thanks to Elsevier Scientific Publishing Company for the permission to reproduce articles originally appearing in *Analytical Chimica Acta* and *Inorganica Chimica Acta*.

REFERENCES

1. J. Raulin, *Annals Sci. natu. botan. biol. végét.* 11(1869)93.
2. G. Lechartier and F. Bellamy, *Compt. Rend. Acad. Sci.* 84(1877)687.
3. F. Raoult and H. Breton, *Compt Rend. Acad. Sci.* 85(1877)40.
4. G. Bertrand and R.C. Bhattacharjee, *Compt. Rend. Acad. Sci.* 198(1934)1823.
5. W.R. Todd, C.A. Elvehjem and E.B. Hart, *Am. J. Physiol.* 107(1934)145.
6. A.S. Prasad, J.A. Halstead and M. Nadimi, *Am. J. Med.* 31(1961)532.
7. A.S. Prasad, A. Miale Jr, Z. Farid, H.H. Sandstead and W.J. Darby, *Anch. Intern. Med.* 111(1963)407.
8. A.S. Prasad, A. Miale, Z. Farid, A. Schubert and H.H. Sandstead, *J. Lab. Clin. Med.* 61(1963)537.
9. J.A. Halstead, H.A. Ronaghy, P. Abadi, H.M. Haghshenass, G.H. Anirhakimi, R.M. Barakat and J.G. Reinhold, *Ann. J. Med.* 53(1972)277.
10. H.A. Ronaghy, J.G. Reinhold, M. Mahaloudji, P. Ghavami, M.R.S. Fox and J.A. Halstead, *Am. J. Clin. Nutr.* 27(1974)112.
11. B.L. Vallee, *Physiol. Rev.* 39(1959)443.
12. *Trace Elements in Human and Animal Nutrition*, E.J. Underwood, ed., 4th ed. Academic Press, New York, 1977.
13. *Trace Elements in Human Health and Disease*, A.S. Prasad, ed., Academic Press, New York, 1976.
14. R.J.P. Williams, *Endeavour* 8(1984)65.
15. D. Keilin and T. Mann, *Biochem. J.* 34(1940)1163.
16. B.L. Vallee, *Adv. Prot. Chem.* 10(1955)317.
17. A. Galdes and B.L. Vallee in *Metal Ions in Biological Systems*, vol. 15, (H. Sigel, ed) Marcel Dekker, New York 1983, p. 1.
18. M. Chvapil, C.F. Zukoski, B.G. Hattler, L. Stankova, D. Montgomery, E.V. Carlson and J.C. Ludwig in *Trace Elements in Human Health and Disease* (A.S. Prasad, ed) Academic Press, New York, 1977, p. 269.
19. M. Chvapil, *Life Sci.* 13(1973)1041.
20. M. Chvapil, *Med. Clin. North Am.* 60(1976)799.
21. H. Rubin, *Proc. Nat. Acad. Sci., USA* 69(1972)712.
22. J.F. Riordan, *Med. Clin. North Am.* 60(1976)661.
23. M.W. Terhune and H.H. Sandstead, *Science* 177(1972)68.
24. M.C. Scrutton, C.W. Wu and D.A. Goldthwait, *Proc. Nat. Acad. Sci. USA*, 68(1971)2497.
25. A.S. Prasad and D. Oberleas, *J. Lab. Clin. Med.* 82(1973)461.
26. J.M. Hsu, in *Trace Elements in Human Health and Disease*, (A.S. Prasad, ed.), Academic Press, New York, 1977, p. 295.
27. A.S. Prasad, *Ann. Rev. Pharmacol. Toxicol.* 20(1979)393.
28. D. McMahon, *Science* 185(1974)1012.
29. *Lancet* i, (1978)191.
30. E.R. Arguilla, S. Packer, W. Tarmas and S. Miyamoto, *Endocrinology* 103(1978)1440.
31. M. Kirchgessner, H.P. Roth and E. Weigand, in *Trace Elements in Human Health and Disease* (A.S. Prasad, ed.) Academic Press, New York, vol. 1, 1976, p. 189.
32. F.K. Habib, *J. Ster. Biochem.* 9(1978)403.
33. T.R. Hartoma, K. Nahoul and A. Netler, *Lancet* 2(1977)1125.

34. L.D. Antoniou, R.J. Shalhoub, T. Sudhakar and J.C. Smith Jr, *Lancet* 2(1977)895.
35. M.P. Macapinlae, W.N. Pearson, G.H. Barney and W.J. Darby, *J. Nutr.* 95(1968)569.
36. M. Somers and E.J. Underwood, *Aust. J. Biol. Sci.* 22(1969)1277.
37. H.H. Madstead, M. Terhune, R.N. Brady, D. Gillespie and W.L. Hollaway, *Clin. Res.* 19(1971)83.
38. G. Ebers, *The Papyrus Ebers: The Great Egyptian Medical Document*; translated by B. Ebbell, Munksgaard, Copenhagen, 1937.
39. E.J. Moynahan, *Bull. Soc. Fr. Derm. Symp.* 80(1973)541.
40. I. Vikbladh, *Scand. J. Clin. Invest.* 3, suppl. 2(1951)1.
41. J.A. Halstead and J.C. Smith Jr, *Lancet* 1(1970)322.
42. P.J. Aggett and J.T. Harries, *Arch Dis. Child.* 54(1979)909.
43. K.M. Hambidge, *Phil. Trans. R. Soc., London B* 294(1981)129.
44. *Br. Med. J.* 282(1981)1098.
45. R.S. Pekarek and W.R. Beisel, *Proc. Soc. Exp. Biol. Med.* 138(1971)728.
46. T. Hallbook and H. Hedelin, *Br. J. Surg.* 64(1977)271.
47. I. Tengrup and H. Samuelson, *Acta Chir. Scand.* 143(1977)195.
48. B.E. Walker, S. Hughes, A.V. Simmons and G.N. Chandler, *Eur. J. Clin. Invest.* 8(1978)193.
49. L.D. McBean, J.C. Smith Jr, B.H. Berne and J.A. Halsted, *Clin. Chim. Acta* 50(1974)43.
50. J. Versiek, F. Barbier, A. Speecke and J. Hoste, *Clin. Chem.* 21(1975)578.
51. W.E.C. Wacker, D.D. Ulmer and B.L. Vallee, *N. Engl. J. Med.* 255(1956)449.
52. R.D. Lindeman, A.A. Yunice, D.J. Baxter, *J. Lab. Clin. Med.* 81(1973)194.
53. A.M. Handjani, J.C. Smith Jr, J.B. Herrmann and J.A. Halsted, *Chest.* 65(1974)185.
54. J. Lekakis and A. Kalotoutis, *Clin. Chem.* 26(1980)1660.
55. C.A. Gervin, W.M. Nichols, M. Chvapil and S.L. Wangenstein, *J. Surg. Res.* 35(1983)340.
56. K.H. Falchuk, *N. Engl. J. Med.* 296(1977)1129.
57. J.A. Halsted, B. Hackley, C. Rudzki and J.C. Smith, Jr, *Gastroenterology* 54(1968)1098.
58. J.D. Boyett and J.F. Sullivan, *Metabolism* 19(1970)148.
59. B.L. Vallee and W.E.C. Wacker, *N. Engl. J. Med.* 257(1957)1055.
60. R.E. Burch, D.A. Sackin and M.M. Jetton, *Gastroenterology* 67(1974)781.
61. J.D. Bogden and R.A. Troiano, *Clin. Chem.* 24(1978)1553.
62. J.B. Freeman, L.D. Stegink, P.D. Meyer, L.K. Fry and L. Devbenten, *J. Surg. Res.* 18(1975)463.
63. A.M. Kahn, H.L. Helwig, A.G. Redeker and T.B. Reynolds, *Am. J. Clin. Pathol.* 44(1965)426.
64. B.E. Walker, J.B. Dawson, J. Kelleher and M.S. Losowsky, *Gut.* 14(1973)943.
65. R. Giroux, P.J. Schechter and J. Schoun, *Clin. Sci. Mol. Med.* 51(1976)545.
66. C.J. Condon and R.M. Freeman, *Ann. Int. Med.* 73(1970)531.
67. M. Cohanin and E.R. Yendt, *The Johns Hopkins Med. J.* 136(1975)137.
68. D.J. Mahler, J.R. Walsh and G.D. Haynie, *Am. J. Clin. Pathol.* 56(1971)17.

69. J. Blomfield, J. McPherson and C.R.D. George, *Br. Med. J.* 2(1969)141.
70. P.A. Simkin, *Lancet* 2(1976)539.
71. Z. Baloch, A.F. El-Chobarey, G.S. Fell, D.H. Brown, J. Dunlop and W.C. Dick, *Ann. Rheum. Dis.* 39(1980)329.
72. R.I. Henkin and F.R. Smith, *Lancet* 1(1971)823.
73. R.I. Henkin and F.R. Smith, *Am. J. Med. Sci.* 264(1972)401.
74. R.I. Henkin, *N. Engl. J. Med.* 291(1974)675.
75. B.O. Beng, Y.K. Kit, M.W. Greaves and V.M. Plummer, *Br. Med. J.* 2(1974)531.
76. A. Flynn, W.J. Pories, W.H. Strain and O.A. Hill Jr, *Lancet* 1(1973)789.
77. I.J. Davies, M. Musa and T.L. Dormandy, *J. Clin. Pathol.* 21(1968)263.
78. International Commission on Radiological Protection. Report of the Task Group on Reference Man, Pergamon Press, Oxford, 1975.
79. R.E. Thiers and B.L. Vallee, *J. Biol. Chem.* 226(1957)911.
80. R.A. McCance and E.M. Widdawson, *Biochem. J.* 36(1942)692.
81. H.M. Tribble and F.I. Scoular, *J. Nutr.* 52(1954)209.
82. H. Spencer, B. Rosoff, I. Lewin and J. Samachson, in *Zinc Metabolism* (A.S. Prasad, ed.) Thomas, Springfield, Ill., 1966, p. 339.
83. W.M. Becker and W.G. Hoekstra in *Intestinal Absorption of Metal Ions, Trace Elements and Radionuclides* (S.C. Skoryna and D. Waldron-Edward, ed.), Pergamon, Oxford, 1971, p.229.
84. B.L. Vallee, R.G. Fluharty and J.G. Givson, *Acta Unio. Intern. Contra Cancrum* 6(1949)869.
85. J.P. Feaster, S.L. Hausard, J.T. McCall, F.H. Skipper and G.K. Davis, *J. Anim. Sci.* 13(1954)781.
86. I.G.F. Gilbert and D.M. Taylor, *Biochim. Biophys. Acta* 21(1956)545.
87. J.C. Heath and J. Liguiner-Milward, *Biochim. Biophys. Acta* 5(1950)404.
88. C. Hahn and G.W. Evans, *Proc. Soc. Exp. Biol. Med.* 144(1973)793.
89. B.C. Starcher, *J. Nutr.* 97(1969)321-
90. D.R. Van Campen and T.J. Kowalski, *Proc. Soc. Exp. Biol. Med.* 136(1971)294.
91. G.W. Evans and C.J. Hahn, *Advan. Exp. Med. Biol.* 48(1974)285.
92. F.A. Suso and H.M. Edwards Jr, *Proc. Soc. Exp. Biol. Med.* 137(1971)306.
93. G.W. Evans, C.I. Grace and H.J. Votava, *Am. J. Physiol.* 228(1975)501.
94. G.W. Evans and T.W. Winter, *Bioch. Biophys. Res. Comm.* 66(1975)218.
95. K.T. Smith and R.J. Cousins, *J. Nutr.* 110(1980)316.
96. J.K. Chesters and M. Will, *Br. J. Nutr.* 46(1981)111.
97. D.M. Foster, R.L. Aamodt, R.I. Henkin and M. Berman, *Am. J. Physiol.* 237(1979)340.
98. H. P. Wolff, *Klin. Wochenschr.* 34(1956)409.
99. A.F. Parisi and B.L. Vallee, *Biochemistry* 9(1970)2421.
100. E.L. Giroux and R.I. Henkin, *Biochim. Biophys. Acta* 273(1972)64.
101. A.S. Prasad and D. Oberleas, *J. Lab. Clin. Med.* 76(1970)416.
102. B.J. Scott and A.R. Bradwell, *Clin. Chem.* 29(1983)629.
103. J.W. Foote and H.T. Delves, *Analyst.* 108(1983)492.

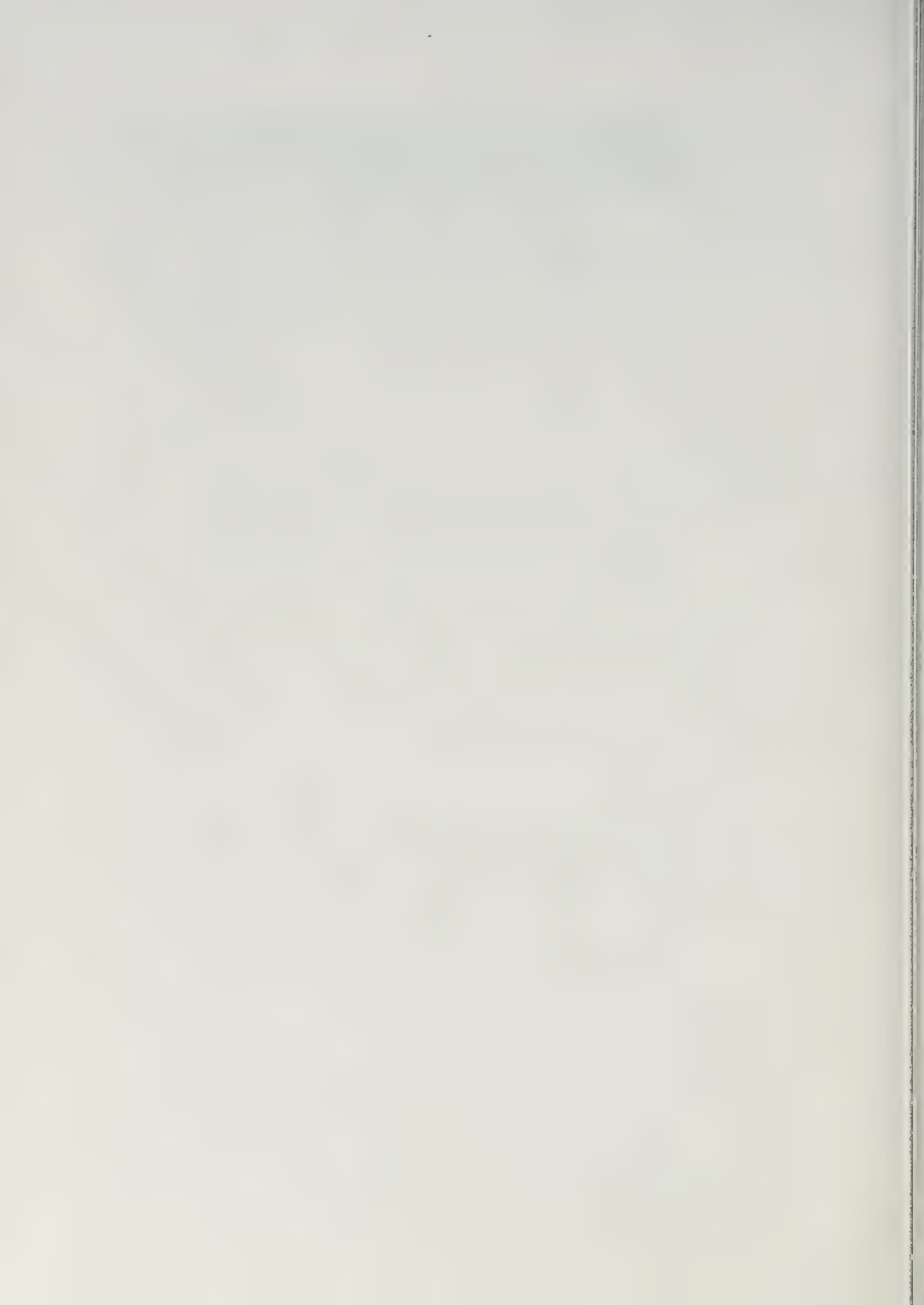
104. D.C. Chilvers, J.B. Dawson, M-H. Bahreyni-Toosi and A. Hodgkinson, *Analyst* 109(1984)871.
105. P.E. Gardiner, J.M. Ottaway, G.S. Fell and R.R. Burns, *Anal. Chim. Acta* 124(1981)281.
106. W.T. Morgan, *Biochim. Biophys. Acta* 533(1978)319.
107. S.L. Guthans and W.T. Morgan, *Arch. Biochem. Biophys.* 218(1982)320.
108. H. Ansell, *Lancet* 1(1839)222.
109. F. Friedberg, *FEBS Lett.* 59(1975)140.
110. T. Peters in *The Plasma Proteins* (F.W. Putman, ed.) Academic, New York, 1975, vol. 1, p.133.
111. B. Meloun, L. Moravek and V. Kostka, *FEBS Lett.* 58(1975)134.
112. P.O. Behrens, A.M. Spiekerman and J.R. Brown, *Fed. Proc.* 34(1975)591.
113. V.M. Rosenoer, M. Oratz and M.A. Rothschild (eds.) *Albumin: Structure, Function and Uses*, Pergamon, New York, 1977.
114. T. Peters and I. Sjoholm (eds.) *Albumin: Structure, Biosynthesis and Function*, Pergamon, New York, 1978.
115. A.A. Spector, *J. Lipid. Res.* 16(1975)165.
116. J.R. Brown, *Fed. Proc.* 34(1975)591.
117. D.D. Van Slyke and G.M. Meyer, *J. Biol. Chem.* 12(1912)399.
118. A.A. Yunice, R.W. King Jr, S. Kraikitpanitch, C.C. Haygood and R.D. Lindeman, *Am. J. Physiol.* 235(1978)40.
119. D.K. Abu-Hamdan, S.D. Migdal, R. Whitehouse, P. Rabbani, A.S. Prasad and F.D. McDonald, *Am. J. Physiol.* 241(1981)487.
120. A.M. van Rij, P.J. Godfrey and J.M. McKenzie, *J. Surg. Res.* 26(1979)293.
121. H. Sigel (ed.) *Metal Ions in Biological Systems*, Marcel Dekker, New York, 1973, vol. 2.
122. D.R. Williams, *The metals of Life*, Van-Nostrand-Reinhold, London, 1971.
123. P.S. Hallman, D.D. Perrin and A.E. Watt, *Biochem. J.* 121(1971)549.
124. G. Berthon, C. Matuchansky and P. May, *J. Inorg. Biochem.* 13(1980)63.
125. E.L. Giroux, *Biomed. Med.* 12(1975)258.
126. P.K. Hall and R.C. Roberts, *Biochem. J.* 171(1978)27.
127. H.E. Schultze, I. Gollner, K. Heide, M. Schonenberger and G. Schwick, *Z. Naturforsch.* 106(1955)463.
128. N. Heimbürger, K. Heide, H. Haupt and H.E. Schultze, *Clin. Chim. Acta* 10(1964)293.
129. J.T. Dunn and R.G. Spiro, *J. Biol. Chem.* 242(1967)5549-
130. R. Bourrillon and E. Kazatimahako in *Glycoproteins* (A. Gotschalk, ed.) Part A, vol. 5, Elsevier, Amsterdam, 1972, p. 699.
131. N.F. Adham, M.K. Song and H. Kinderknecht, *Biochim. Biophys. Acta* 495(1977)212.
132. P. Wilding, N.F. Adham, J.W. Mehl and B.J. Haverback, *Nature (London)* 214(1967)1226.
133. L. Sottrup-Jensen, T.M. Stepanik, T. Kristensen, D.M. Wierzbicki, C.M. Jones, P.B. Lonblad, S. Magnusson and T.E. Petersen, *J. Biol. Chem.* 259(1984)8318.
134. M.K. Song and N.F. Adham, *Clin. Chim. Acta* 99(1979)13.

135. B. Sarkar and T.P.A. Kruck in *The Biochemistry of Copper* (J. Peisach, P. Aisen and W.E. Blumberg, eds.) Academic Press, New York, 1966, p. 183.
136. C. Whitehouse, A.S. Prasad and Z.T. Cossack, *Clin. Chem.* 29(1983)1974.
137. E. Dennes, R. Tupper and A. Wormall, *Biochem. J.* 82(1962)466.
138. S.R. Himmelhoch, H.A. Sober, B.L. Vallee, E.A. Peterson and K. Fuwa, *Biochemistry* 5(1966)2523.
139. J.B. Dawson, M.H. Bakereyni-Toosi, D.J. Elki and A. Hodgkinson, *Analyst* 106(1981)153.
140. J.R. Kelson, *Clin. Chem.* 22(1976)1218, abs.
141. F. Alt and P. Burba, *Z. Anal. Chem.* 317(1984)656.
142. M. Stoeppler and H.W. Nürnberg, in *The Use of Biological Specimens for the Assessment of Human Exposure to Environmental Pollution* (A. Berlin, A.H. Wolff and Y. Hasegawa, eds.), M. Nijhoff, The Hague, 1979, p. 325.
143. D. Behne and H. Jürgensen, *J. Radional. Chem.* 42(1978)447.
144. I.G. Stump, J. Carruthers, J. M. D'Auria, D.A. Applegarth and A.G.F. Davidson, *Clin. Biochem.* 10(1977)127.
145. W. Niedermeier and J.H. Griggs, *J. Chronic. Dis.* 23(1971)527.
146. S.A. Clyburn, G.F. Seriv, B.R. Bartchmid, J.E. Evans and C. Veillon, *Analyt. Biochem.* 63(1975)231.
147. H.W. Nürnberg, in *Analytical Techniques for Heavy Metals in Biological Fluids* (S. Facchetti, ed.) Elsevier, Amsterdam, 1981, p. 209.
148. L. Gorton and U. Fiedler, *Anal. Chim. Acta* 90(1977)233.
149. L. Risinger, L. Ögren and G. Johansson, *Anal. Chim. Acta* 154(1983)251.
150. F. Bloch, *Phys. Rev.* 70(1946)460.
151. E.M. Purcell, H.C. Torrey and R.V. Pound, *Phys. Rev.* 69(1946)37.
152. A. Abragam, *The Principles of magnetic resonance*, Clarendon, Oxford, 1961.
153. W.D. Knight, *Phys. Rev.* 76(1949)1259.
154. M. Eigen, *Pure Appl. Chem.* 6(1963)97.
155. J.E. Coleman and J.F. Chlebowski, *Advances in Inorganic Biochemistry* (Eichorn and Mazilli, eds.), Elsevier, Amsterdam, 1979, p. 1.
156. J.E. Coleman, I.M. Armitage, J.F. Chlebowski, J. P. Otvos and A.J.M.S. Uiterkamp, in *Biological Applications of Magnetic Resonance* (R.G. Schulman, ed.), Academic, New York, 1979, p. 345.
157. I.M. Armitage, R.T. Pajer, A.J.M.S. Uiterkamp, J.F. Chlebowski and J. Coleman, *J. Am. Chem. Soc.* 98(1976)5710.
158. J.L. Sudmeier and S.T. Bell, *J. Am. Chem. Soc.* 99(1977)4499.
159. E.O. Martins and T. Drakenberg, unpublished.
160. J. Bogden and M.M. Joselow, *Am. Ind. Hyg. Assoc. J.* 35(1974)88.
161. R.A.A. Muzzarelli and R. Rochetti, *Talanta* 22(1975)683.
162. P. del Castilho and R.F.M. Herber, *Anal. Chim. Acta* 94(1977)269.
163. S. Kiilerich, M.S. Christensen, J. Naestoft and C. Christensen, *Clin. Chem. Acta* 105(1980)231.
164. A. Taylor and T.N. Bryant, *Clin. Chim. Acta* 110(1981)83.
165. J. Verrieck, A. Speecke, J. Hoste and J. Barbier, *Z. Klin. Chem. Klin. Biochem.* 11(1973)193.

166. R.M. Wheeler, R.B. Liebert, T. Zabel, R.P. Chaturvedi, V. Valkovic, G.C. Philips, P.S. Ong, E.L. Cheng and M. Hrgoveic, *Med. Physics* 1(1974)68.
167. P. Carter, *Clin. Chim. Acta* 52(1974)277.
168. M. Ujiie, I. Mikami, T. Gemba, A. Nomura, M. Tokudome and K. Fujii, *Clin. Chim. Acta* 87(1978)71.
169. I. Sinko and S. Gomiscek, *Mikrochim. Acta* (1972)163.
170. P. Valenta, L. Mart and H. Rutzel, *J. Electroanal. Chem.* 82(1977)327.
171. M.L. Failla and R.J. Cousins, *Biochim. Biophys. Acta* 538(1978)435.
172. N.H. Stacey and C.D. Klaassen, *Biochim. Biophys. Acta* 640(1981)693.
173. M. Chvapil, J.N. Ryan and C.F. Zukoski, *Proc. Soc. Exper. Biol. Med.* 140(1972)642.
174. L. Warren, M.C. Glick and M.K. Nass, *J. Cell. Physiol.* 68(1966)269.
175. M.C. Powanda, G.L. Cockerell and R.S. Pekarek, *Am. J. Physiol.* 225(1973)399.
176. G.F. Fuhrmann and A. Rothstein, *Biochim. Biophys. Acta* 163(1968)325.
177. W.C. Kruckeberg and G.J. Brewer, *Med. Biol.* 56(1978)5.
178. K.T. Smith, M.L. Failla and R.J. Cousins, *Biochem. J.* 184(1978)627.
179. S. Kowarski, C.S. Blair-Stanek and D. Schachter, *Am. J. Physiol.* 266(1974)401.
180. F.J. Schwarz and G. Matrone, *Proc. Soc. Exp. Biol. Med.* 149(1975)888.
181. R.I. Henkin, B.M. Patten, P.K. Re and D.A. Bronzert, *Arch. Neurol.* 32(1975)745.
182. J. Horcicko, J. Borovansky, M. Kubikova, J. Duchon and H. Duchkova, *Clin. Chim. Acta* 104(1980)377.
183. J.D. Bogden, R.A. Troiano and M.M. Joselow, *Clin. Chem.* 23(1977)485.
184. P.W.N. Keeling, R.B. Jones, P.J. Hilton and P.H. Thompson, *Gut.* 21(1980)561.
185. L. Ellis, *Clin. Chim. Acta* 82(1978)105.
186. T. Graham, *Phil. Trans. Roy. Soc.* 151(1861)1836.
187. T. Graham, *Phil. Mag.* 32(1866)402.
188. W.J. Kolff and H.T. Berk, *Acta Med. Scand.* 117(1944)121.
189. H.K. Lonsdale, *J. Membr. Sci.* 10(1982)81.
190. E.A. Guggenheim, *Thermodynamics*, North-Holland, Amsterdam, 1950.
191. H.I. Mahon and B.J. Lipps, *Encycl. Polym. Sci. Technol.* 15(1971)258.
192. L.C. Craig, *Science* 144(1964)1093; *Methods in Enzymol.* 11(1967)870.
193. K.K. Steward, *Adv. Prot. Chem.* 31(1977)135.
194. R.B. Dean, *Chem. Rev.* 41(1947)503.
195. H.H. Stein, *Anal. Biochem.* 13(1965)305.
196. S.P. Colowick and F.C. Womak, *J. Biol. Chem.* 244(1969)774.
197. M. Hashimoto, T. Higashi, A. Isomoto, M. Vozumi and A. Okumura, *Anal. Biochem.* 137(1984)344.
198. K. Feldmann, *Anal. Biochem.* 88(1978)225.
199. J. Toffaletti, J. Savory and H.J. Gitelman, *Clin. Chem.* 23(1977)1258.

200. W.E. van der Linden, *Anal. Chim. Acta* 151(1983)359.
201. Y.M. Fraticelli and M.E. Meyerhoff, *Anal. Chem.* 55(1983)359.
202. L. Gorton and L. Ögren, *Anal. Chim. Acta* 130(1981)45.
203. E.L. Cussler, *Diffusion Mass Transfer in Fluid Systems*, Cambridge UP, Cambridge, 1984.
204. J.M. Kooijman, *Chem. Eng. Sci.* 28(1973)1149.
205. E.J. Davies, *Can. J. Chem. Eng.* 51(1973)562.
206. D.O. Cooney, E.J. Davies and S-S. Kim, *Chem. Eng. J.* 8(1974)213.
207. D.O. Cooney, S-S. Kim and E.J. Davies, *Chem. Eng. Sci.* 29(1974)1731.
208. J.A. Blanco and W.N. Gill, *Chem. Eng. Progr. Symp. Ser.* 63(1967)66.
209. R.J. Nunge, E.W. Porta and W.N. Gill, *Chem. Eng. Progr. Symp. Ser.* 63(1967)80.
210. L.D. McBean, J.C. Smith Jr, B.H. Berne and J.A. Halsted, *Clin. Chim. Acta* 50(1974)43.
211. M.F. Re and D.N. Bennion, *Ind. Eng. Chem. Fundam.* 12(1973)296.
212. K.W. Choi and D.N. Bennion, *Ind. Eng. Chem. Fundam.* 14(1975)296.
213. D.N. Bennion and B.W. Rhee, *Ind. Eng. Chem. Fundam.* 8(1969)36.
214. J.C. Anderson and J.A. Quinn, *Biophys. J.* 14(1974)130.
215. A.J. Staverman, *Rev. Trav. Chim.* 70(1951)344.
216. K.S. Spiegler, *Trans. Faraday Soc.* 54(1958)1408.
217. P.H. Barry and J.M. Diamond, *Phys. Rev.* 64(1984)763.
218. O. Kedem and A. Katchalsky, *Biophys. J.* 2(1962)530.
219. T.K. Sherwood, P.L.T. Brian and R.E. Fisher, *Ind. Eng. Chem. Fundam.* 6(1967)2.
220. C.V. Paganelli and A.K. Solomon, *J. Gen. Physiol.* 41(1957)259.
221. T. Matsuura, L. Pagean and S. Sourirajan, *J. Appl. Polym. Sci.* 19(1975)179.
222. H.K. Lonsdale, U. Merten and R.L. Riley, *J. Appl. Polym. Sci.* 9(1965)1341.
223. S.T. Hwang, T.E.S. Tang and K. Kammermeyer, *J. Macromol. Sci. Phys. B.* 5(1971)1.
224. E.E. Wilson, *Trans. Am. Soc. Mech. Eng.* 37(1915)47.
225. T.G. Kaufmann and E.F. Leonard, *AIChEJ.* 14(1968)421.
226. M.P. Bohrer, *Ind. Eng. Chem. Fundam.* 22(1983)72.
227. A.L. Babb, C.J. Maurer, D.L. Fry, R.P. Popovich and R.E. McKee, *Chem. Eng. Progr. Symp. Ser.* 84, 64(1968)59.
228. K.A. Smith, C.K. Colton, E.W. Merrill and L.B. Evans, *Chem. Eng. Progr. Symp. Ser.* 84, 64(1968)45.
229. C.K. Colton and K.A. Smith, *AIChE J.* 18(1972)958.
230. D.M. Malone and J.C. Anderson, *AIChE J.* 23(1977)177.
231. V.G. Levich, *Physicochemical Hydrodynamics*, Prentice-Hall, Englewood Cliffs, N.J., 1962.
232. M.E. Heyde, C.R. Peters and J.E. Anderson, *J. Cell. Interf. Sci.* 50(1975)467.
233. M.E. Heyde and J.E. Anderson, *J. Phys. Chem.* 79(1975)1659.
234. E. Glueckauf, *Desalination* 18(1976)155.
235. T.D. Hodgson, *Desalination* 8(1970)99.
236. R.A. Robinson and R.H. Stokes, *Electrolyte Solutions*, Butterworths, London, 1970.
237. D.G. Leaist and P.A. Lyons, *J. Phys. Chem.* 85(1981)1756.
238. L.C. Craig and W. Konigsberg, *J. Phys. Chem.* 65(1961)166.
239. L.C. Craig nad T.O. King, *J. Am. Chem. Soc.* 77(1955)6620.
240. L.C. Craig and A. Ansevin, *Biochemistry* 2(1963)1268.

- 241. G.S. Barker and I.L. Jenkins, *Analyst* 77(1952)685.
- 242. E. Barendrecht, in *Electroanalytical Chemistry* (A.J. Bard, ed.), vol. 2, Marcel Dekker, New York, 1967, p. 53.
- 243. D.R. Crow, *Polarography of Metal Complexes*, Academic, London, 1969.
- 244. W. Davison, *J. Electroanal. Chem.* 87(1978)395.





Cadmium(II), Zinc(II), and Copper(II) Ions Binding to Bovine Serum Albumin. A ^{113}Cd NMR Study

EDUARDO O. MARTINS and TORBJÖRN DRAGENBERG

Departments of Analytical Chemistry and Physical Chemistry 2, University of Lund, POB 740, S-220 07 Lund 7, Sweden

Received February 2, 1982

The interaction between bovine serum albumin and the metals Cd(II), Zn(II) and Cu(II) was studied by ^{113}Cd NMR. A ^{113}Cd titration of the protein revealed two strong Cd(II) binding sites. Competition between Zn(II) (or Cu(II)) and Cd(II) for the strong sites indicates that there is a common strong binding site for Cu(II), Zn(II) and Cd(II), with higher affinity for Cu(II) and Zn(II) than for Cd(II). Competition between serum albumin and NTA for Cd(II) did not allow conclusive evaluation of the intrinsic binding constants, probably due to the formation of ternary complexes NTA–Cd(II)–protein. Chemical shifts are in good agreement with histidyl residue involvement in one site, and probably oxygen ligands in a second site. The site responsible for strong binding of Zn(II) contains two histidyl residues, but not thiol groups.

Introduction

The importance of serum albumin as the main carrier of metal ions in the blood is well established [1]. Transition metal interaction with albumin attracted wide interest in the past. In most of these studies, however, it has been assumed that several identical binding sites exist. Binding constants for these sites have been obtained using various methods. Binding of zinc, for instance, has been subject to numerous studies [2–15], but as far as we have found in the literature only two attempts have been made to determine the binding constant for specific high-affinity sites. Österberg [11] has found that there is one high affinity site whose intrinsic binding constant can be determined only when the pK of the displaced proton is known. Constants with values ranging from $\text{p}K_a = 9.6$ and downward can be calculated with the highest value where the thiol group is involved. From a competition between serum albumin and various amino acids for zinc ions, Giroux and Henkin [12] obtained a binding constant $\text{p}K_a = 6.98$.

Metal NMR has for some time been used to study metal binding to small ligands, as well as to proteins. ^{113}Cd NMR has proven to be very suitable for such studies, especially because cadmium can replace both zinc, in several enzymes [16], and calcium in structural calcium binding proteins [17]. We therefore

decided to study metal binding to serum albumin by means of ^{113}Cd NMR, and in this work we will present a study on cadmium binding to serum albumin, as well as the competition between cadmium and zinc (or copper).

Experimental

Crystalline bovine serum albumin was obtained from Sigma (A-6003, fraction V, essentially fatty acid free). Solutions of the protein in 0.16 M NaCl (Merck, Supra pur.) were dialyzed against EDTA and subsequently dialyzed several times against distilled water. Cadmium, zinc and copper solutions were prepared from the respective perchlorate salts.

NMR measurements

The ^{113}Cd NMR measurements have been performed on a home-made spectrometer [18] using a 6T Oxford superconducting magnet, giving a ^{113}Cd resonance frequency of 56.55 MHz. Normally 2 mM albumin samples have been used, with varying concentrations of cadmium and other metal ions. 100,000 transients with a flip angle of ca. 45° (8 μs) required ca. 8 h of accumulation. The samples were in 17 mm O.D. ampoules inserted horizontally. The ^{113}Cd NMR chemical shifts are given relative to that of an aqueous 0.1 M $\text{Cd}(\text{ClO}_4)_2$ solution, with high frequency (low field) shifts taken as positive.

Results and Discussion

Cd binding

The addition of $^{113}\text{Cd}(\text{II})$ ions to a 2 mM solution of serum albumin gives rise to two ^{113}Cd NMR signals, with chemical shifts at +150 ppm and +28 ppm. Both resonances show excessive broadening, at 700 Hz and 450 Hz, respectively. The broadening is not dependent on the temperature, which indicates that it is not due to metal exchange. Furthermore the lack of temperature dependence shows that the life-time of the bound cadmium ions is $>10^{-2}$ s. Figure 1 shows that the two strong Cd(II) sites ($\delta = +150$ and +28 ppm) have similar binding constants since the

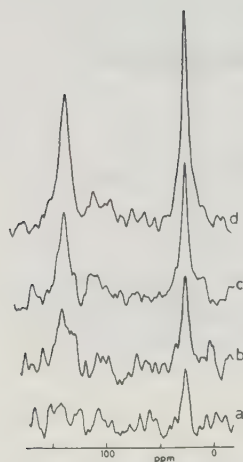


Fig. 1. ^{113}Cd NMR spectra showing the effect of increasing amounts of $^{113}\text{Cd}(\text{II})$ to a 2 mM serum albumin solution. pH 6.7, 0.16 M NaCl. a) 1 mM Cd(II), b) 2 mM Cd(II), c) 3 mM Cd(II), d) 4 mM Cd(II).

intensities of the two resonances increase almost in parallel with increasing cadmium concentrations. When an excess of cadmium (more than two equivalents per albumin molecule) is used, a very broad ^{113}Cd NMR signal at about +80 ppm appears. This signal is temperature dependent and broadens beyond detection at +8 °C. This indicates that this resonance is due to ^{113}Cd ions with a lifetime on the protein site of ca. 10^{-5} s.

Titration shows that the two strong Cd(II) sites ($\delta = +150$ ppm and +28 ppm) are pH dependent, in the range 5–7. Above pH 7 the two sites appear insensitive to pH changes, and below pH 5 only one ^{113}Cd signal with a chemical shift (of +80 ppm) is observed. The present data cannot be used to calculate the cadmium–serum albumin binding constant. We have, however, tried to estimate it in two different ways.

At first, the mere fact that the exchange of cadmium from two sites on serum albumin is sufficiently slow to give rise to resolvable, non-exchange broadened resonances, indicates that the binding constants for these sites are large. Assuming that the Cd(II) on-rate is diffusion limited, $k_{\text{on}} \sim 10^9 \text{ s}^{-1}$ (this is an assumption which we have no proof for; however, it is often assumed that the metal on-rate is diffusion limited [19], but there are cases where the on-rate is significantly slower than the diffusion limit [20]) then, we find $K_{\text{a}} > 10^7 \text{ M}^{-1}$ for these two strong sites, using the above estimated lower limit for the lifetime, 10^{-2} s. Similarly, the binding constant for

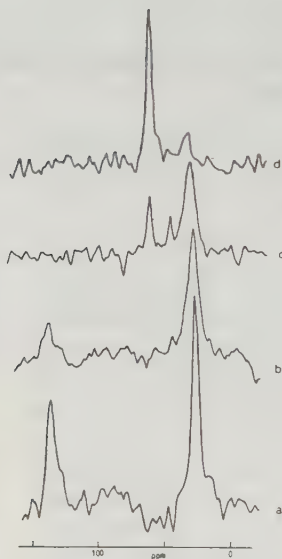


Fig. 2. ^{113}Cd NMR spectra (4 mM Cd(II) and 2 mM serum albumin, pH 5.7) showing the effect of addition of NTA. a) No NTA, b) 2 mM NTA, c) 6 mM NTA, d) 12 mM NTA.

the weaker sites giving rise to the resonance observed (+80 ppm) can be estimated to $K_{\text{a}} \approx 10^4 \text{ M}^{-1}$.

Secondly, we tried to perform a competition experiment using NTA (nitriloacetate). As shown in Fig. 2, the ^{113}Cd spectrum is drastically affected by the addition of NTA. However, the disappearance of the ^{113}Cd signal at +150 ppm is not followed by the appearance of a new signal of the Cd(NTA) or Cd(NTA)₂ complexes, which is around +10 ppm (unpublished results). Sarkar and Wigfield [21] have shown that ternary complexes can be formed between serum albumin, Cu(II) and amino acids (histidine and threonine). An obvious explanation to our results is therefore that mixed complexes can also be found between serum albumin, Cd(II) and NTA, even though the complexing properties of Cu(II) and Cd(II) are different. The ^{113}Cd NMR signal observed at +60 ppm could thus be tentatively assigned to the ternary complex. Moreover the absence of a signal corresponding to Cd(NTA)₂, even when a large excess of NTA is present, shows that the ternary complex must be stronger than Cd(NTA)₂. These observations corroborate the above estimated K_{a} value for the two strong cadmium sites. The ^{113}Cd NMR chemical shifts of the two strongly bound cadmium ions indicate that there is no sulphur involved in the cadmium binding, since it has been shown that sulphur binding results in chemical shifts which are of several hundred ppm

higher frequency than those observed here [22]. The +150 ppm shift agrees quite well with that found for the enzymes carboxypeptidase, alkaline phosphatase and superoxide dismutase, where the cadmium ion is supposed to be bound to two or three histidyl residues [16]. There are very few ^{113}Cd chemical shifts for protein bound cadmium in the range 0–100 ppm, which makes it difficult to assign (even tentatively) the binding site corresponding to +28 ppm. However, the fact that there is a competition, although weak, with Ca(II) for this site indicates that only oxygen is involved in the binding of the metal ions.

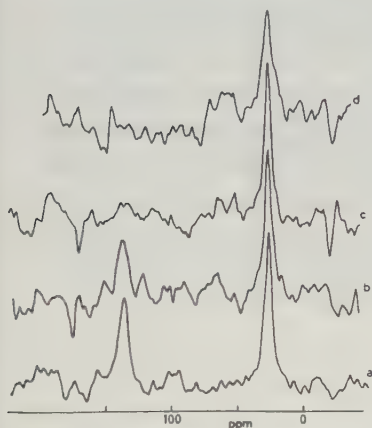


Fig. 3. ^{113}Cd NMR spectra (4 mM Cd(II) , 2 mM serum albumin, pH 7.5) showing the effect of addition of Zn(II) . a) No Zn(II) , b) 1 mM Zn(II) , c) 2 mM Zn(II) , d) 4 mM Zn(II) .

Metal competition

In order to determine if any of the two strong Cd(II) sites are the same as the Zn(II) or Cu(II) sites, which are normally assumed to be different, we made two competition experiments adding either Zn(II) or Cu(II) ions to the Cd(II) –serum albumin solution. In Fig. 3, the Zn(II) competition is shown. It is obvious that the ^{113}Cd site with a shift of ca. +150 ppm is the same as the strong Zn(II) site. The competition with Cu(II) (not shown) gave much the same results as for the Zn(II) . The ^{113}Cd signal with a shift of $\sim +150$ ppm disappeared upon the addition of 1 equiv. of Cu(II) ; with an excess of Cu(II) , the second ^{113}Cd signal became slightly broadened. We can state positively that there is a common, strong binding site for Cu(II) , Zn(II) and Cd(II) . This site has a higher affinity for Cu(II) and Zn(II) than for Cd(II) . There is also a second strong Cd(II) site which does not bind either Zn(II) or Cu(II) strongly. It might be worth mentioning that the so called exchangeable copper

[23] cannot be that bound to the strong site with a $K_a = 10^{16}$, since this will give (assuming a diffusion limited on-rate) an off-rate of ca. 10^{-7} s^{-1} , or a lifetime of ca. 3,000 hours. The competition between Ca(II) or Gd(III) with Cd(II) did not give conclusive results. For Ca(II) there is only a minor effect on the ^{113}Cd resonances, even when a 4-fold excess of Ca(II) was added. The intensities of the two signals were slightly reduced, the high field signal (+28 ppm) showing the largest decrease. With Gd(III) both ^{113}Cd signals are affected upon the addition of less than one equivalent of Gd(III) per albumin molecule. However, there is no strong selectivity towards one particular site, at most a slight preference for the site with the high field shifted ^{113}Cd resonance. With two Gd(III) equivalents only a broad ^{113}Cd signal, with a shift of about +80 ppm, could be observed.

Our present results strongly support the conclusion by Giroux and Henkin [12] that an overwhelming proportion of the zinc in normal serum is bound to serum albumin. The site responsible for Zn(II) binding consists most probably of two histidyl residues and not the cysteinyl residue. This site also appears to be the strong Cu(II) site. In all the zinc metalloenzymes so far studied for which X-ray data are available, the active zinc site involves two or three histidyl residues [24]. In metamyoglobin, moreover, it was shown that one equivalent of Zn(II) is bound to a nearby position, probably also involving a histidyl residue. In solution Zn(II) competes with Cu(II) for some of the sites. For serum albumins X-ray data are not yet available, but from amino acid sequence [25, 26] and secondary structure prediction [27] it is possible to recognize only one region, the N-terminus, where two histidyl residues (positions 3 and 10) are sufficiently close to participate in the binding of the same metal ion. For serum albumin, the binding of the first Cu(II) equivalent has been thoroughly studied and one strong site was located at the N-terminus of the peptide chain, involving the histidyl residue in position 3 [18–32], which agrees with our findings that both Zn(II) and Cu(II) replace the same Cd(II) from its binding site. This does not necessarily mean that the binding sites for these three ions are identical but that, e.g., the binding of Cu(II) to its site excludes Zn(II) or Cd(II) from their sites.

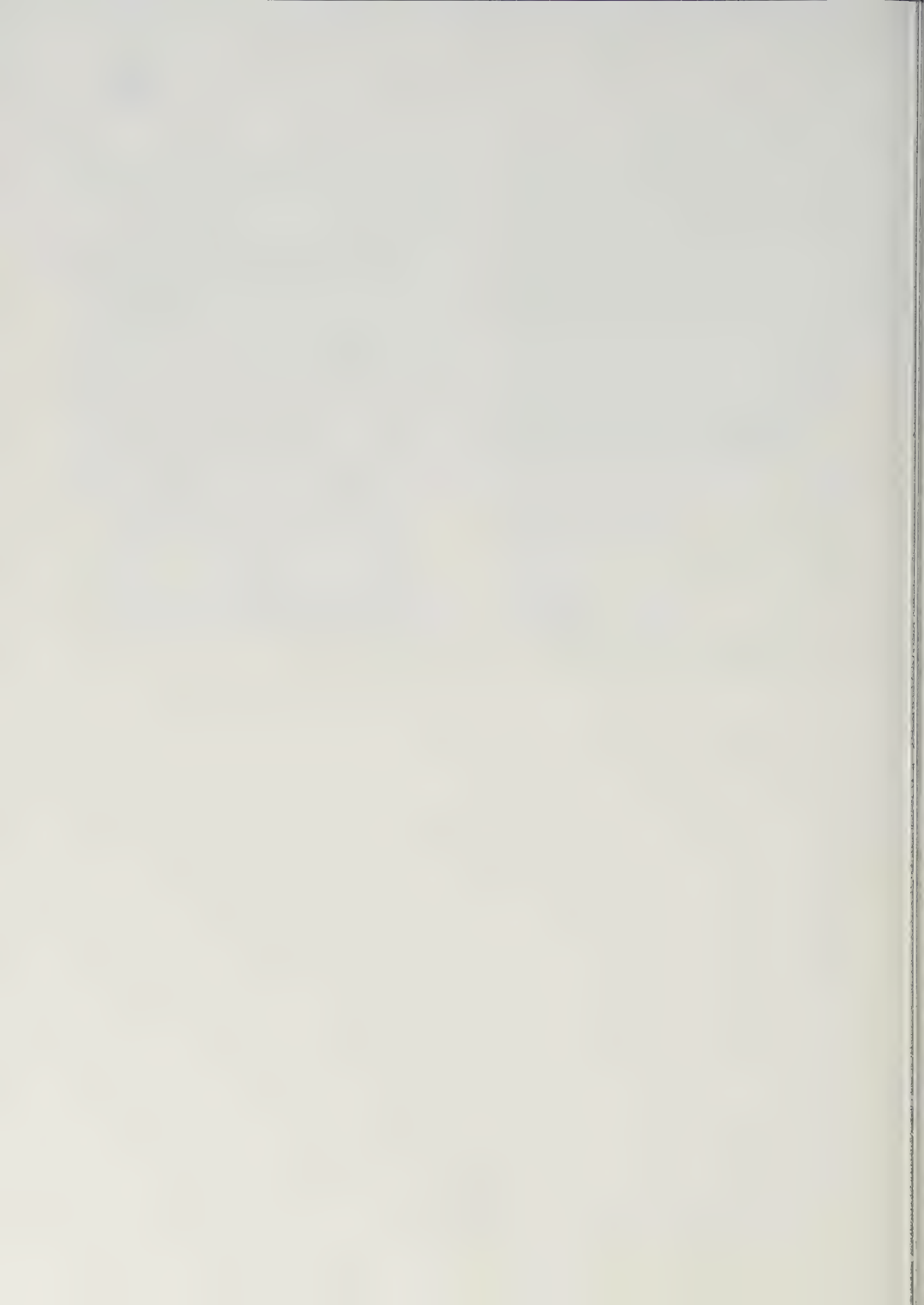
A second strong Cd(II) binding site, which also binds Ca(II) but not as strongly as Cd(II) , might be a site where the metal ion is bound only to oxygen in less than octahedral geometry.

Acknowledgement

This work was made possible by grants from the Swedish Natural Sciences Research Council.

References

- 1 F. Friedberg, *FEBS Lett.*, **59**, 140 (1975).
- 2 C. Tanford, *J. Am. Chem. Soc.*, **73**, 2066 (1951).
- 3 C. Tanford, *J. Am. Chem. Soc.*, **74**, 211 (1952).
- 4 F. R. N. Gurd and D. J. Goodman, *J. Am. Chem. Soc.*, **74**, 671 (1952).
- 5 H. A. Saroff and H. J. Mark, *J. Am. Chem. Soc.*, **75**, 1420 (1953).
- 6 H. Lal and M. S. N. Rao, *J. Am. Chem. Soc.*, **79**, 3050 (1957).
- 7 M. S. N. Rao and H. Lal, *J. Am. Chem. Soc.*, **80**, 3222 (1958).
- 8 M. S. N. Rao and H. Lal, *J. Am. Chem. Soc.*, **80**, 3226 (1958).
- 9 H. Waldmann-Meyer, *J. Biol. Chem.*, **235**, 3337 (1960).
- 10 D. J. Perkins, *Biochem. J.*, **80**, 668 (1961).
- 11 R. Österberg, *Acta Chem. Scand.*, **25**, 3827 (1971).
- 12 E. L. Giroux and R. I. Henkin, *Biochim. Biophys. Acta*, **273**, 64 (1972).
- 13 Y. Hashimoto, *Chem. Abstr.*, **89**: 101237a (1977).
- 14 A. M. Fiabane, M. L. D. Touche and D. R. Williams, *J. Inorg. Nucl. Chem.*, **40**, 1201 (1978).
- 15 H. Waldmann-Meyer, *Anal. Chem. Symp. Ser.*, **3**, 125 (1980).
- 16 I. M. Armitage, A. J. M. S. Uiterkamp, J. F. Chlebowski and J. E. Coleman, *J. Magn. Res.*, **29**, 375 (1978).
- 17 S. Forsén and B. Lindman, 'Ion Binding in Biological Systems as Studied by NMR Spectroscopy', in 'Methods of Biochemical Analysis' (D. Glick, ed.), New York (1981).
- 18 S. Forsén, E. Thulin and H. Lilja, *FEBS Lett.*, **104**, 123 (1979).
- 19 J. D. Johnson, S. C. Charton and J. D. Potter, *J. Biol. Chem.*, **254**, 3497 (1979).
- 20 T. Anderson, T. Drakenberg, S. Forsén, T. Wieloch and M. Lindström, *FEBS Lett.*, **123**, 115 (1981).
- 21 B. Sarkar and Y. Wigfield, *Can. J. Biochem.*, **46**, 601 (1968).
- 22 R. J. Kostelnik and A. A. Bothner-By, *J. Magn. Res.*, **14**, 141 (1974).
- 23 S. J. Lau, T. P. A. Kruck and B. Sarkar, *J. Biol. Chem.*, **249**, 5878 (1974).
- 24 B. Sarkar, in 'Metal Ligand Interaction in Organic and Bio-Chemistry' (B. Pullman, ed.), Dordrecht (1977).
- 25 J. R. Brown, *Federation Proc.*, **35**, 2141 (1976).
- 26 B. Meloun, L. Morávek and V. Kostka, *FEBS Lett.*, **58**, 134 (1975).
- 27 A. D. McLachlan and J. E. Walker, *Biochim. Biophys. Acta*, **536**, 107 (1978).
- 28 R. A. Bradshaw, W. T. Shearer and F. R. N. Gurd, *J. Biol. Chem.*, **243**, 3817 (1968).
- 29 R. A. Bradshaw and T. Peters, Jr., *J. Biol. Chem.*, **244**, 5582 (1969).
- 30 P. Z. Neumann and A. San-Kortsak, *J. Clin. Invest.*, **46**, 446 (1967).
- 31 T. Peters, Jr., and F. A. Bluenmenstock, *J. Biol. Chem.*, **242**, 1574 (1967).
- 32 W. T. Shearer, R. A. Bradshaw, F. R. N. Gurd and T. Peters, Jr., *J. Biol. Chem.*, **242**, 5451 (1967).



MODEL EXPERIMENTS FOR DETERMINATIONS OF ZINC–AMINO ACID COMPLEXES IN BIOLOGICAL FLUIDS BY POLAROGRAPHY

EDUARDO O. MARTINS and GILLIS JOHANSSON*

Department of Analytical Chemistry, University of Lund, P.O. Box 740, S-220 07 Lund (Sweden)

(Received 12th November 1979)

SUMMARY

The Ilkovič constant was determined for zinc and zinc–amino acid complexes by using differential pulse polarography in 0.1 M sodium chloride–sodium diethylbarbiturate buffer at pH 7.4. A single well-behaved wave was obtained, except for cysteine and cystine for which useful calibration curves could not be obtained. The non-equilibrium dialysis of the amino acid complexes was studied in a hollow-fiber dialyzer. It is concluded that the dialysis step could be used in a method intended to measure available zinc but that the polarographic method needs further modification.

Normal zinc concentration in human serum is about 1 mg l^{-1} , one third of which is strongly bound to globulin, mainly α -2-macroglobulin, and the remainder is loosely bound, principally to albumin. A smaller fraction, ca. 2% of the total, is complexed by amino acids. It is generally accepted that the non-protein-bound fraction of zinc may have an important role in the transport of this element. Amino acids, especially cysteine, histidine, glutamine, cystine and glycine, have been recognized as the principal ligands [1, 2]. Giroux and Henkin [2] concluded that amino acids compete effectively with albumin for binding of zinc, histidine and cysteine being the most successful. In physiological concentrations, addition of amino acids to predialyzed serum increased the ultrafiltrable ^{65}Zn several fold [1], histidine, glutamine, threonine, cystine and lysine showing the most marked effects.

Several pathological conditions provoke a redistribution of plasma zinc within body tissues [3]; in particular, it has been suggested that in the course of acute viral hepatitis, virtually all the detectable zinc exists in an ultrafiltrable state in low-molecular-weight complexes [4]. Also, amino acid-bound zinc may account for most of the urinary zinc in normal and pathological situations [5].

Determination of total zinc concentrations, e.g. by atomic absorption, does not provide information about the proportions present in the complexed and free forms. Present knowledge about available zinc has been obtained either by using complex-forming agents like dithizone or zincon followed by spectrophotometry, or by dialysis or ultrafiltration followed by various methods for analysis of the dialysate. Practically nothing has

been done to develop methods which can be used for routine determinations of available zinc in clinical samples. Even for research purposes the currently available methods are inadequate.

The prospects of finding methods for direct determination of available zinc in a serum sample without previous separation of proteins do not seem to be good at present. An ion-selective electrode was developed with this purpose in mind [6, 7] but neither sensitivity nor selectivity was sufficient for serum analysis. Direct use of voltammetric methods is out of the question as sulfhydryl and disulfide groups in the proteins adsorb on and react with mercury and other metals. The resulting waves are complicated and dependent on uncontrollable parameters, and it is generally impossible to use the method analytically.

This paper describes a study of a model system comprising a microdialyzer to prevent proteins from reaching the polarographic electrode. Continued work to develop a method for available zinc is in progress.

EXPERIMENTAL

Polarographic measurements were made with a PAR 174A polarograph equipped with a PAR drop timer. A pulse height of 50 mV, a drop time of 1 s and a scan rate of 2 mV s^{-1} were used throughout. A saturated calomel electrode was used as the reference and a platinum wire as the auxiliary electrode. Solutions were degassed with purified nitrogen which had been deoxygenated over copper turnings at 500°C and equilibrated with the supporting electrolyte. During the measurements, nitrogen was passed over the solution. The solutions were thermostatted at $25.0 \pm 0.2^\circ\text{C}$.

The dialyzer was a flow-through hollow-filter microdialyzer (AB Gambro, P.O. Box 10015, S-220 10 Lund, Swedish patent 396819). When not in use it was soaked with glycerine to prevent bacterial growth and stored in a refrigerator. The microdialyzer was checked for copper contamination; the leakage of copper was found to be less than the detection limit, $10^{-7} \text{ M Cu}^{2+}$. The solutions were pumped with an Ismatec MP 13 GJ-4 multichannel peristaltic pump.

In the dialysis experiments, the synthetic sample was pumped through the outer tubing at a constant rate of 6.08 or 6.23 ml min^{-1} . It has previously been shown for glucose [8] that the flow rate in the outer tubing has no influence on the dialysis constant K if the flow is greater than 4.7 ml min^{-1} . The flow rate through the inner tubing, which was mounted coaxially inside the outer, was pumped in the opposite direction at the rates specified in the Results section. Steady state was attained within 5 min for the lowest inner-tubing flow, 0.09 ml min^{-1} .

Fractions of the inner-tube flow were collected and transferred to the polarographic cell for analysis.

The buffer used throughout was 0.10 M NaCl — 1 mM sodium diethylbarbiturate adjusted to pH 7.4. All chemicals were of analytical-reagent

grade. The amino acids were obtained from Sigma. Solutions were prepared with water purified by a Millipore-Q system. Zinc contamination from reagents and laboratory ware could be kept below the detection limit, 10^{-7} M Zn^{2+} .

RESULTS AND DISCUSSION

Polarography of amino acid complexes

The reduction of zinc by polarographic techniques has been studied by several authors [9–11]. The reduction in buffer solutions occurs with different degrees of irreversibility. It is well known (see e.g. [12, 13]) that the zinc–amino acid complexes give two waves in unbuffered solutions. In this study a buffer containing sodium chloride and a barbiturate was selected because the waves of both zinc and the amino acid complexes behaved very well. In this particular buffer, the calibration graphs for both the metal and its complexes were strictly linear.

Table 1 summarizes the result of measurements with differential pulse polarography. Various metal and amino acid concentrations were tested. A mixture of seven amino acids taken in the proportion normally found in serum was also studied. In all cases a single wave was obtained and the peak potential was the same for the aquo ion as for all the complexes. The value of the Ilkovič constant, I_p/C , varied within $\pm 28\%$ from the mean value of 1.56×10^{-5} M μA^{-1} . The reproducibility was very good. Several waves were produced by solutions containing both zinc and cysteine or/and cystine. The pattern of the polarograms was dependent on both the pH and the concentrations. There was one peak at -0.985 V as for the other zinc–amino acid complexes, but it was distorted and there was another peak in the cathodic direction which partly overlapped the first one. There were no linear calibration graphs within the serum concentration range. Even in a mixture, the presence of these two amino acids interfered.

The detection limit was 1×10^{-7} M Zn^{2+} if dissolved oxygen was thoroughly removed. Because of the cell construction, degassing for 80–90 min was necessary at this level.

Dialysis experiments

For linear dialysis, the concentration of the solute, C_e , in the dialysate should be

$$C_e = AD C_s / bf_i = K C_s \quad (1)$$

where A is the membrane area, b its thickness, D the diffusion coefficient of the solute, f_i the inner-tubing flow rate and C_s the solute concentration in the sample. For a given experimental arrangement with constant flow rates, all parameters can be compounded to a new constant, K , and C_e should be a linear function of C_s . It has been shown by Gorton and Bhatti [8] that the Gambro hollow-fiber dialyzer was almost linear for the dialysis of

TABLE 1

Differential pulse polarography of zinc and zinc-amino acid (AA) solutions in 0.10 M NaCl-1 mM Na-diethylbarbiturate, pH 7.4

$[Zn^{2+}]_T$ (mM)	$[AA]_T$ (mM)	E_p (V vs. SCE)	I_p/C ($\times 10^{-5}$ M μA^{-1})
0.0050-0.024	—	-0.985	1.19
0.0010-0.024	GLY, 0.0010	-0.985	1.23
—	GLY, 0.0100	-0.985	1.24
—	GLY, 0.100	-0.985	1.25
—	GLY, 1.00	-0.987	1.31
—	GLY, 10.0	-0.987	1.29
—	ALA, 0.100	-0.985	1.43
—	ALA, 1.00	-0.985	1.43
0.0010-0.015	GLU, 0.910	-0.985	1.14
0.0010-0.050	GLU, 10.0	-0.985	1.12
0.0010-0.012	HIS, 0.131	-0.985	1.99
—	ARG, 0.0120	-0.985	1.79
—	ARG, 0.107	-0.985	1.82
0.00010-0.060	LYS, 0.150	-0.982	1.86
—	THR, 0.148	-0.985	1.82
0.00010-0.060	GLY, 0.300	-0.987	1.77
	GLU, 0.700		
	HIS, 0.120		
	LYS, 0.150		
	ALA, 0.350		
	ARG, 0.100		
	THR, 0.150		

glucose, resulting in a value of $Kf_i = 0.0472$ ml min⁻¹. The value of Kf_i is expected to be different for each solute, depending partly on different values of D but to a greater extent on differences in the interaction between the membrane and the solute molecules. This interaction is not explicitly included in eqn. (1), but for charged solute and charged membranes the effect may be very pronounced. In addition, there is a cut-off value for transport through the membrane which for the present dialyzers is somewhere above 1000 Daltons. It was shown by Gorton and Bhatti [8] that less than 0.01% (the detection limit) of serum albumin at physiological concentrations passes through the membrane.

Table 2 shows a test of dialyzer linearity for zinc-glycine complexes with glycine in excess, for two different dialyzers. Regression analysis showed that the slopes, b , are very close to zero indicating that the constant K in eqn. (1) is independent of C_s . Table 3 shows that Kf_i is the same for the two dialyzers. For substantially lower flow rates, the constant Kf_i decreases. The reason for this was not investigated but in experiments not tabulated it was shown that a small change in the outer-tubing flow rate had no effect whatsoever. It was also found that the dialysis was linear with concentration C_s at the lower flow rate.

TABLE 2

Dialysis of zinc from a solution 10 mM in glycine and 0.10 M in NaCl

Outer tubing		Inner tubing		Flow rate 0.964 ml min ⁻¹			
Flow rate		Flow rate 0.755 ml min ⁻¹		Dialyzer I		Dialyzer II	
6.083 ml min ⁻¹		Dialyzer I	Dialyzer II	[Zn-GLY] dialyzed (mM)		[Zn-GLY] dialyzed (mM)	
[Zn ²⁺] (mM)		[Zn-GLY] dialyzed (mM)	K(Zn-GLY) ^a	[Zn-GLY] dialyzed (mM)	K(Zn-GLY) ^b	[Zn-GLY] dialyzed (mM)	K(Zn-GLY) ^d
0.040		0.00080	0.0199	0.00070	0.0175	0.00060	0.0162
0.200		0.00400	0.0198	0.00320	0.0158	0.00340	0.0170
0.400		0.00770	0.0193	0.00660	0.0164	0.00670	0.0167
1.00		0.0215	0.0215	0.0189	0.0189	0.0165	0.0165
2.00		0.0406	0.0203	0.0330	0.0165	0.0312	0.0156
3.00		0.0613	0.0204	0.0495	0.0165	0.0488	0.0163
4.00		0.0804	0.0210	0.0650	0.0164	0.0650	0.0162

^a_a = 0.0002 mM, _b = 0.0202, $r = 0.9998$. (_a = intercept; _b = slope; r = correlation coefficient). _b_a = 0.0003 mM, _b = 0.0205, $r = 0.9997$.
_c_a = 0.0005 mM, _b = 0.0164, $r = 0.9993$. _d_a = 0 mM, _b = 0.0162, $r = 0.9998$.

TABLE 3

Dialysis parameters of two dialyzers for zinc-glycine solutions

Flow rate (ml min ⁻¹)		Dialyzer I		Dialyzer II	
Outer tubing	Inner tubing	$K(\text{Zn-GLY})$	$K \times f_i$ (ml min ⁻¹)	$K(\text{Zn-GLY})$	$K \times f_i$ (ml min ⁻¹)
6.08	0.755	0.0202	0.0152	0.0205	0.0155
6.08	0.964	0.0164	0.0158	0.0162	0.0156

Table 4 shows the values of K and Kf_i for zinc and for different zinc-amino acid complexes. Each value is the mean of five series; for each series, five different zinc concentrations were tested. It can be seen that these constants are higher for zinc ions than for the complexes. This is to be expected because the larger complexes should have smaller diffusion coefficients. The range of K for the complexes is 0.104–0.119 if the large outlying value for arginine is excluded. The standard deviation obtained for each value was 5–6%. The single outlying value for arginine was probably due to a change in the pump rate of a defective tubing. A somewhat better reproducibility should have been obtained if the dialyzer had been thermostatted.

A synthetic amino acid mixture with a composition near that of a normal serum gave a K value of 0.106. If the mixture is used as a calibration solution, a systematic error of at most 13% will result if a single amino acid dominates completely.

From the long-term stability of dialyzer I, shown in Table 5, it can be concluded that the device can be used, at least for synthetic solutions, with some confidence.

Prospects for application to serum analysis

The model experiments discussed above show that the dialysis of the various amino acid complexes occurs at almost the same rate. At most an error of 13% should result if a particular amino acid predominates when a mixture has been assumed. The free zinc is dialyzed more rapidly but this is of no practical importance as there is always an excess of amino acids in a real sample.

The polarographic data, however, showed a maximum deviation of –37% from a value obtained in the model mixture. This is of course unacceptable as a basis for an analytical method. Furthermore, cysteine and cystine interfere strongly and they are always present in a real sample. Even if differential pulse polarography is out of the question, other polarographic methods might be useful. Preliminary measurements by anodic stripping voltammetry from zinc-cysteine complexes have shown that the interferences can be overcome. Also the differences between the response factors of the various ligands are smaller than in differential pulse polarography.

TABLE 4

Results from dialysis experiments and dialysis parameters for dialyzer I. Zinc—amino acid (AA) solutions in 0.10 M NaCl, dialyzed against the buffer, at constant inner-tubing flow rate, 0.090 ml min⁻¹.

[Zn ²⁺] _T (mM)	[AA] _T (mM)	K(Zn—AA)	K × f _i (ml min ⁻¹)	[Zn ²⁺] _T (mM)	[AA] _T (mM)	K(Zn—AA)	K × f _i (ml min ⁻¹)
0.010—4.00	—	0.136	0.0122	0.010—0.60	GLY, 0.300	0.106	0.0095
0.010—0.60	GLY, 6.00	0.111	0.0100		GLU, 0.600		
0.010—1.00	GLY, 10.0	0.116	0.0104		HIS, 0.120		
0.010—1.00	GLY, 1.00	0.114	0.0103		LYS, 0.150		
	ALA, 0.350	0.104	0.0094		ALA, 0.350		
	ALA, 1.00	0.119	0.0107		ARG, 0.100		
	GLU, 1.00	0.115	0.0103		THR, 0.150		
	GLU, 1.00	0.112	0.0100				
0.010—0.60	GLU, 0.60	0.116	0.0104				
	ARG, 0.094	0.122	0.0110				
	ARG, 0.118	0.118	0.0106				
	HIS, 0.115	0.116	0.0104				
	THR, 0.148	0.104	0.0093				
	LYS, 0.15	0.106	0.0095				

TABLE 5

Dialysis parameters for dialyzer I. Zinc solutions, 0.02 mM in 0.10 M NaCl, dialyzed against the buffer at two different inner-tubing flow rates.

Inner tubing flow rate, f _i (ml min ⁻¹)	K(Zn ²⁺)	K × f _i (ml min ⁻¹)
0.090	0.136	0.0122
0.964	0.0131	0.0126
0.090 ^a	0.135	0.0122
0.964 ^a	0.0128	0.0123

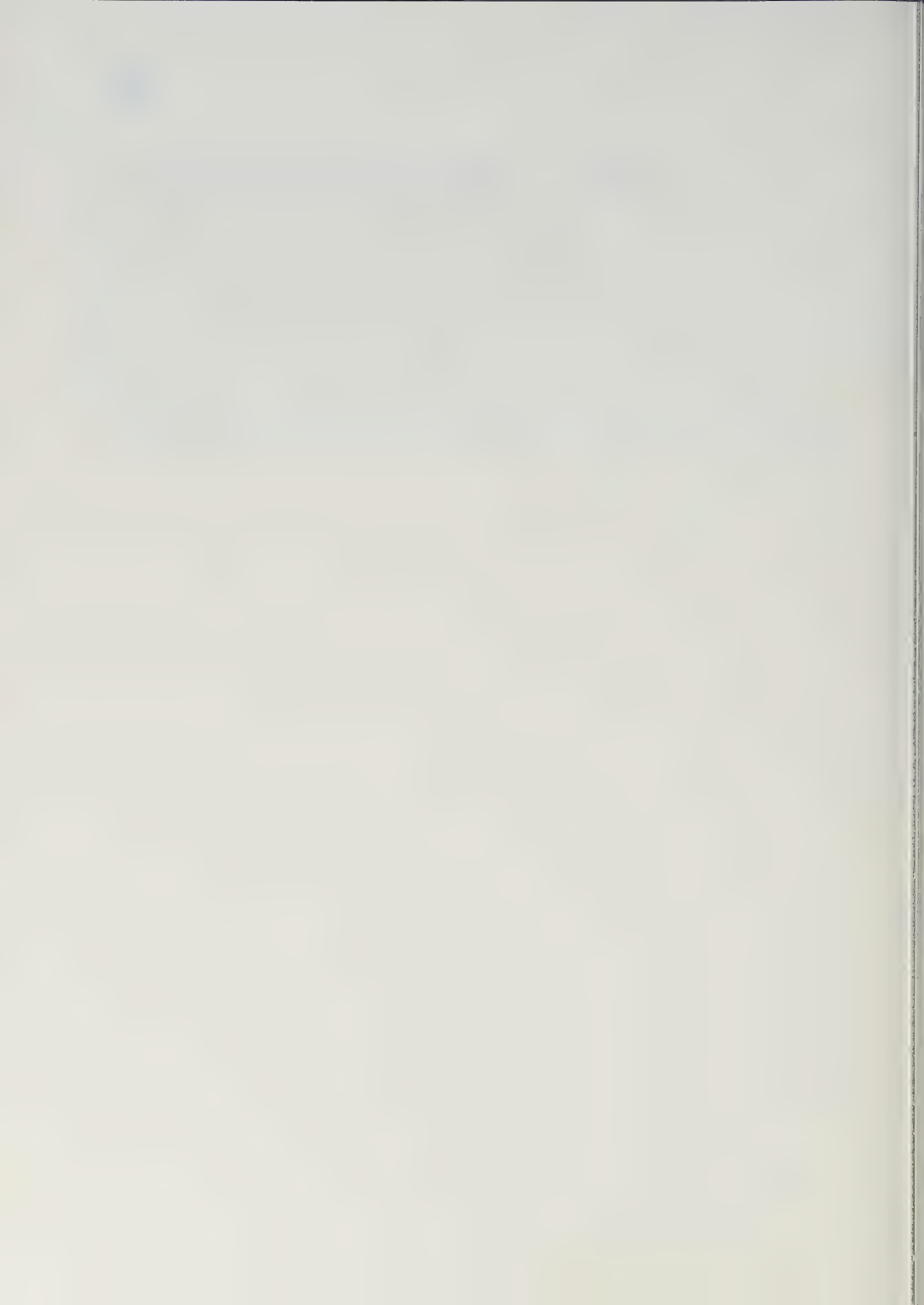
^aAfter five months of experiments.

Polarographic methods have some attractive properties when compared, for example, to atomic absorption analysis of the dialysate. Only complexes with a reasonable rate of ligand exchange will be measured by polarographic method. It is known that there are some zinc complexes in the body with extremely slow exchange rates. If a measure of available zinc is required, a method which can differentiate between zinc bound to fast-exchanging and slow-exchanging ligands should be of considerable interest.

This work was supported by grants from the Swedish Natural Science Research Council. The authors thank Ulla Fiedler-Linnersund for valuable discussions.

REFERENCES

- 1 A. S. Prasad and D. Oberleas, *J. Lab. Clin. Med.*, 76 (1970) 416.
- 2 E. L. Giroux and R. I. Henkin, *Biochim. Biophys. Acta*, 273 (1972) 64.
- 3 W. R. Beisel, *Med. Clin. North Am.*, 60 (1976) 831.
- 4 R. I. Henkin and F. R. Smith, *J. Med. Sci.*, 264 (1972) 401.
- 5 R. I. Henkin, H. R. Keiser and D. Bronzert, *J. Clin. Invest.*, 51 (1972) 44a.
- 6 L. Gorton and U. Fiedler, *Anal. Chim. Acta*, 90 (1977) 233.
- 7 U. Fiedler-Linnersund and K. M. Bhatti, *Anal. Chim. Acta*, 111 (1979) 57.
- 8 L. Gorton and K. M. Bhatti, *Anal. Chim. Acta*, 105 (1979) 43.
- 9 Y. Ayabe, *J. Electroanal. Chem.*, 55 (1974) 187.
- 10 J. H. Christie, E. P. Parry and R. A. Osteryoung, *Electrochim. Acta*, 11 (1966) 1525.
- 11 J. Koryta, *Electrochim. Acta*, 6 (1962) 67.
- 12 R. Sundaresan, S. C. Saraiya and A. K. Sundaram, *Proc. Indian Acad. Sci.*, 66A (1967) 184.
- 13 R. Sundaresan, S. C. Saraiya, T. P. Radhakrishnan and A. K. Sundaram, *Electrochim. Acta*, 13 (1968) 443.



A FLOW-THROUGH CELL FOR DIFFERENTIAL PULSE ANODIC STRIPPING VOLTAMMETRY

EDUARDO O. MARTINS and GILLIS JOHANSSON*

Department of Analytical Chemistry, University of Lund, P.O. Box 740, S-220 07 Lund (Sweden)

(Received 23rd February 1982)

SUMMARY

A flow-through voltammetric cell with a hanging mercury drop electrode has been developed to fit the static mercury drop electrode (PAR 303). The design has resulted in a linear increase of sensitivity with flow rate and an enhancement of sensitivity by the wall-jet effect. The cell is used in a flow injection system in which samples are introduced with a Růžička–Hansen injector. The mercury drop is held at plating potentials while the sample peak passes through the cell. Stripping is done under stopped flow conditions, to reduce noise, after the sample has been washed completely from the cell. The stripping thus takes place into the carrier electrolyte which always has a constant composition independent of sample constituents. Film-forming interfering species will, however, remain on the surface of the mercury drop. The effect of medium exchange on films produced by L-cysteine is reported. The flow-through medium exchange simplifies deaeration, speeds up analysis and reduces contamination.

The increasing demand for rapid and sensitive methods for continuous determinations of organic and inorganic compounds has led to the development of on-line flow-through voltammetric techniques [1–3]. Much of the work on electrochemical detectors in the last decade has been intended for high-performance liquid chromatography (h.p.l.c.), where low dead volumes and fast responses are essential. For trace metal determinations, anodic stripping becomes the method of choice because of its superior sensitivity. Successful applications have been described for mercury-coated graphite or glassy-carbon flow-through electrodes [4–10] as well as for hanging mercury drop electrodes [11, 12]. The model 303 static mercury drop electrode (SMDE) with the 310 LC-detector (Princeton Applied Research) also provides for anodic stripping in flowing solutions [13].

Mercury-coated carbon electrodes are able to produce great sensitivity and they are easily adapted to various flow-cell designs. The mercury is thought to form small droplets on the surface and this may be one of the reasons why the overpotential is lower than that of a pure mercury surface. There are also various reports about adsorption of organic compounds, surface catalysis and deterioration from poisoning. Interferences from these sources are more pronounced in biological samples such as serum than in

samples dominated by inorganic constituents. A mercury drop electrode is to be preferred in organic matrices, especially for the determination of zinc which strips at high overpotential. The surface of drop electrodes is more easily renewed, which is of importance if the number of samples is large. Zinc determination in, for example, serum samples requires utmost sensitivity if it is to be used as a speciation method. Most of the zinc is complexed with, or incorporated into, the proteins. A small fraction is mobile and can be transferred, e.g., across a dialysis membrane. In a previous study [14], the rate of dialysis of zinc amino acid complexes was measured. A flow-through dialyzer will prevent proteins from interfering with the voltammetric measurements. Smaller molecules will pass, however, and one of the most troublesome of these, cysteine, was included in this study of cell construction and cell performance.

A medium exchange has sometimes been recommended as an alternative to separation methods when sample components interfere with the stripping. The hanging drop is transferred from the sample solution into a pure buffer for stripping. This method has not been much used because it is time-consuming and requires very careful handling. A partial reoxidation of the amalgam, up to 10% [15] may occur during the transfer. Koster and Ariel [11] made a flow cell (not a flow-through cell) in which the drop remained stationary while the solutions were exchanged. Here, it was decided to make the medium exchange by flow injection analysis (f.i.a.).

EXPERIMENTAL

The flow-through cell

A static mercury drop electrode (SMDE, model 303, Princeton Applied Research) was modified for operation with a flow cell. The original nylon block containing the hammer was replaced by another block framed to give firm support to the cell (Fig. 1). For a wall-jet cell to obtain good reproducibility, the position of the capillary has to be precise within 0.1 mm or better, demanding great mechanical stability of the system. The cell is thus screwed into the block as far as a firm stop provided by a planar surface.

The cell itself consists of three parts, all made of plexiglas. A new top can easily be built if a new capillary with a different length is set in operation. The cell top is sealed with a soft rubber membrane in which a hole (about 1 mm) is punched for the capillary. The rubber should be very soft, otherwise the automatic dislodgement of the mercury will be impaired. Unless a soft rubber is used, the capillary will be forced into an asymmetrical position. The rubber membranes were obtained from a dentist who uses them for pressing around teeth during dental operations. The middle part of the cell is machined to fit the auxiliary electrode and the capillary. A platinum ring pressed into the plexiglas serves as the auxiliary electrode. The reference electrode is made from a 0.5 mm platinum wire which has been silvered and chloridated. It is screwed into the side of the cell body, and a salt bridge,

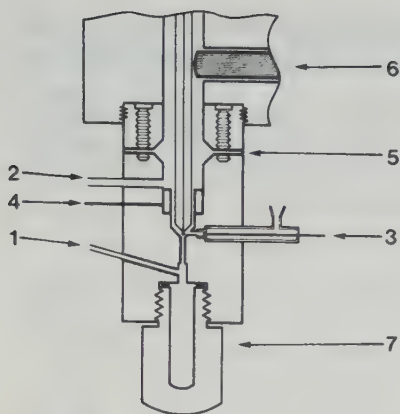


Fig. 1. Flow cell 1, inlet; 2, outlet; 3, reference electrode; 4, auxiliary electrode; 5, membrane; 6, hammer; 7, mercury reservoir.

plugged at the end with asbestos soaked in 0.1 M KCl, serves as a junction. The final part of the salt bridge is only 0.5 mm i.d. to reduce influence on the flow pattern around the mercury drop. The reference solution, 0.1 M KCl, is filled into the reference electrode as needed with a syringe. The potential of the reference electrode was 38.6 mV vs. SCE (theory 40.6). All potential values in this paper have been recalculated so that they refer to SCE.

The main problem to be solved in building a flow cell for a mercury electrode is to collect the used drops without introducing undue dead volumes. Separation in this cell takes place in a wider section (i.d. 2 mm) and the drops fall down into the mercury reservoir at the bottom. The separator, volume 25 μ l, and some exchange with the liquid at the top of the mercury reservoir causes practically all the band broadening from the cell. The conical volume around the mercury drop has a volume of 4 μ l. A small restrictor pressed into the 2-mm hole from below should reduce band-broadening. It was not tried because it was not important for the present application.

All external tubing was of teflon (i.d. 0.3 mm). Thick-walled single tubing was not sufficient to prevent oxygen diffusion into the stream. Double-walled tubings (one tubing being forced into another close-fitting tubing) were adequate. The tubing was connected to the cell with Altex screw fittings (not shown in Fig. 1). All connectors were sealed with small O-rings at the bottom.

Decontamination

Electrolytes were prepared from Suprapur chemicals (Merck, Darmstadt). The water was purified by reverse osmosis followed by a Millipore-Q (ion exchange, absorption, filtration) unit. The carrier electrolyte was pre-

electrolyzed at -1.45 V over a stirred mercury pool for at least 72 h. All glass vessels were soaked in 2 M nitric acid (p.a.) for 2–3 days and rinsed thoroughly with purified water.

The carrier electrolyte, 0.16 M NaCl–50 mM sodium acetate, pH 5.0, was deaerated by nitrogen bubbling in the reservoir. An all-steel nitrogen pressure regulator and steel tubings were used to prevent oxygen contamination. The samples were deaerated briefly before injection.

Instrumental arrangement

A PAR model 174 polarographic analyzer was used with the SMDE, modified as described above. Deoxygenated carrier electrolyte was pumped from the reservoir by an Ismatec MP13 GJ-4 peristaltic pump into a Růžicka–Hansen [16] injection valve and further into the voltammetric cell.

The mercury drops are metered out automatically by the SMDE. Only the largest drop size was used but then the flow rate became limited to less than 3 ml min^{-1} . The drop will almost touch the inlet tube and a high flow rate will cause vibrations in the drop. Smaller drops will not cause the wall-jet effect with the same position of the capillary. It was not possible to align the capillary precisely enough sideways to get a reproducible wall-jet effect with smaller drops.

Flow pulsations induce a high noise level unless the flow is stopped during recording of the stripping voltammogram. If the peristaltic pump is replaced with an h.p.l.c. pump (Waters, Model M-6000A) with a pulse dampener (LDC Model 296) and a restrictor, recordings could be done with low noise level even during flow. Neither the h.p.l.c. pump nor the steel tubings could be used in trace metal analysis because of metal leaching.

A potential of -1.2 V is applied well before the front of a sample entered the cell. This potential is kept constant until the sample has been flushed completely from the cell. The flow is then stopped and the stripping is initiated after a rest period of 10 s. Except where mentioned, the sweep rate was usually 5 mV s^{-1} , the pulse repetition time 0.5 s and the pulse height -50 mV . The injected volumes were 50–500 μl . Two drops of mercury were dislodged before the next sample was injected.

RESULTS

Cell evaluation with continuous sample flow

Zinc standards were pumped continuously through the cell and the plating time was determined as is usual in differential-pulse anodic stripping voltammetry (d.p.a.s.v.), otherwise the procedure was as described in Experimental. The response, i.e., the height of the zinc peak, increased almost linearly with flow rate throughout the investigated range (0.16 – 3.0 ml min^{-1}). The sensitivity changed very little with concentration between 5×10^{-8} and 10^{-6} M zinc(II). Normalized results, obtained by dividing actual currents by the current at 3.0 ml min^{-1} , are shown in Fig. 2, curve A. Earlier versions of

the flow cell gave humps in plots of response vs. flow rate. Even with this cell, the sensitivity will decrease with flow rate at high flow rates (well above 3 ml min^{-1}).

The calibration curves were strictly linear at constant flow rate and constant plating time. The necessary plating times were 6–8 min at $5 \times 10^{-9} \text{ M}$, 2 min at 10^{-8} M and 20 s at 10^{-7} M zinc(II) for a flow rate of 0.66 ml min^{-1} . Plating times longer than 10 min cause peak broadening, because of diffusion into the capillary. The SMDE capillary has a much wider bore than those normally used. The slopes of the calibration curves were 1.99×10^7 , 5.16×10^7 and $1.06 \times 10^8 \mu\text{A M}^{-1}$ for plating times of 2, 5 and 10 min, respectively ($r = 0.9999$ – 0.998).

The sensitivity is enhanced about two-fold by the wall-jet effect. The distance between the drop and the inlet can be increased by spacers between the cell top and the nylon block. Figure 2, curve B, shows that the enhancement disappears completely with a 90.5 mm spacer.

The plating current was measured in the sampled-d.c. mode in an $8 \times 10^{-7} \text{ M}$ zinc(II) solution. The slope became 0.58 in a log–log plot of current vs. flow rate. According to Hanekamp and van Nieuwkerk [17], the slope should be 0.33 for tubular, 0.50 for disk, and 0.75 for true wall-jet electrodes. The observed slope falls between that of a disk and a true wall-jet electrode. The theory applies to plating but not to stripping.

Dispersion

The dispersion in the voltammetric cell in the sampled-d.c. mode was compared to that of a spectrophotometric h.p.l.c. detector (LDC Spectro-Monitor III, cell volume $10 \mu\text{l}$). Samples (50 – $500 \mu\text{l}$) of a catechol solution

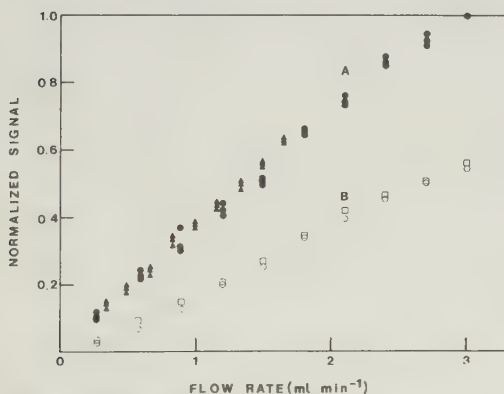


Fig. 2. Dependence of the signal on the flow rate. D.p.a.s.v. mode; plating potential -1.35 V ; pulse -50 mV ; repetition time 0.5 s ; scan rate 5 mV s^{-1} . (A) Distance between the inlet nozzle and the capillary tip, 1.0 mm ; ($\bullet$) $5.0 \times 10^{-8} \text{ M Zn}^{2+}$; ($\blacktriangle$) $9.8 \times 10^{-8} \text{ M Zn}^{2+}$; three plating times. (B) Distance between the inlet nozzle and the capillary tip, 2.0 mm ; ($\square$) 1.5 mm ; ($\circ$) 2.0 mm ; $5.0 \times 10^{-8} \text{ M Zn}^{2+}$.

were injected when the u.v. detector was connected and samples of zinc(II) when the voltammetric cell was connected. The dispersion was 1.8–2.5 greater for the polarographic cell. The dispersion of the latter increased when the flow rate decreased probably because of larger interaction with the solution in the mercury reservoir. The delay between injection and peak maximum was the same but the tailing was much larger for the voltammetric cell. The type of pump had little effect on dispersion except for a slightly lower dispersion at low flow rates with a peristaltic pump. The noise level of the detector was, as noted above, much lower for the h.p.l.c. pump.

The peak height recorded polarographically doubles when the flow rate doubles, whereas the peak height of the u.v. detector is almost independent of changes in the flow.

Suppression of interferences by medium exchange

Some interferences in d.p.a.s.v. can be removed or suppressed by using the flow injection system. The determination of zinc in samples containing cysteine will demonstrate the approach. The complete procedure in d.p.a.s.v. (i.e., both plating and stripping) is normally made in the solution which contains the sample. If the sample contains cysteine (Fig. 3) the zinc peak will

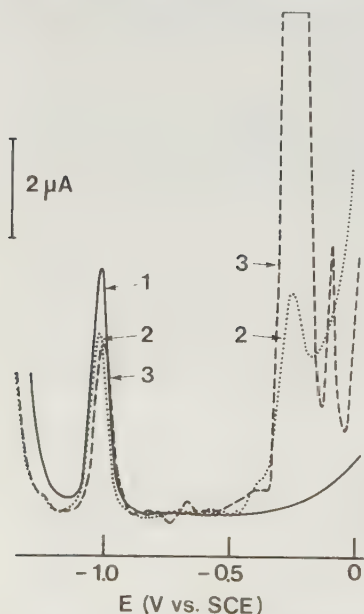


Fig. 3. D.p.a.s.v. of zinc-L-cysteine solutions. Plating potential -1.4 ; pulse -50 mV; repetition time 0.5 s; scan rate 5 mV s^{-1} ; flow rate 1.8 ml min^{-1} ; plating time, 20 s. (1) 6.12×10^{-7} M Zn^{2+} , no L-cysteine, 0.16 M NaCl, 50 mM acetate buffer, pH 5.0 ; (2) 6.12×10^{-7} M Zn^{2+} , 14×10^{-6} M L-cysteine, 0.16 M NaCl, 50 mM acetate buffer, pH 5.0 ; (3) 6.12×10^{-7} M Zn^{2+} , 83×10^{-6} M L-cysteine, 0.16 M NaCl, 50 mM acetate buffer, pH 5.0 .

decrease compared to a sample without this interference. Anodic peaks in the range -0.4 to 0 V vs. SCE will prevent the determination of metals which are stripped below about -0.4 V. The interferences are even worse in direct polarographic methods than in d.p.a.s.v. as the latter method enhances the current from plated metals compared to direct faradaic reactions. Trace metal determinations in biological samples are usually out of the question unless a decomposition pretreatment has been used.

In a flow injection system, however, the plating can be started just before the sample arrives in the cell and continued until the peak has been eluted completely. The cell then contains only carrier electrolyte, all soluble interfering substances in the sample having been washed out. Stripping into pure acetate buffer (Fig. 4) indeed shows that the anodic cysteine peak is lower than when stripping is done into a solution also containing the sample (Fig. 3). The f.i.a. sample peak lasts for about 20 s; comparison of results when the stripping is delayed 2 min (Fig. 4A) or 7 min (Fig. 4B) shows that the L-cysteine desorbs slowly from the HMDE. Yet a second scan after brief flushing with carrier electrolyte (dashed line, Fig. 4B) shows that the film responsible for the peak at -0.3 V remains almost unchanged on the drop. The adsorbed substance can be repeatedly oxidized and reduced. The zinc is stripped out during the first scan, and is not affected by the prolonged wash-out time.

The rate of plating of zinc is, to a first approximation, not affected by the film on the mercury drop, as can be seen in Table 1. The zinc peak is about 14% lower if the sample contains cysteine but this is of the same order as the expected decrease in diffusion rate of the zinc caused by complexation. The data therefore support the rather surprising conclusion that the film which clings to the drop does not affect either the rate of plating or the rate of

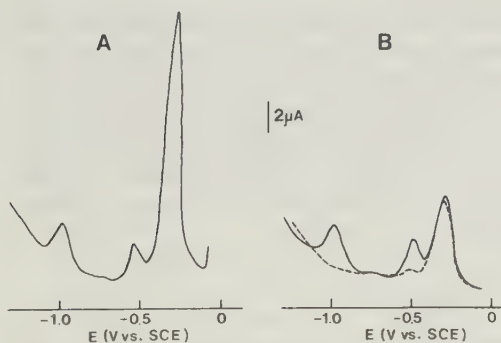


Fig. 4. Influence of medium exchange on the d.p.a.s.v. of zinc-L-cysteine solutions. Plating potential -1.4 V; pulse -50 mV; repetition time 0.5 s; scan rate 5 mV s $^{-1}$; flow rate 1.8 ml min $^{-1}$; 0.16 M NaCl- 50 mM acetate buffer carrier; pH 5.0 . The injected sample is 500 μ l of Zn $^{2+}$ -L-cysteine solution (1.06×10^{-7} M Zn $^{2+}$ and 85×10^{-6} M L-cysteine in 0.16 M NaCl-acetate buffer, pH 5.0). Wash-out time between the plating and stripping steps: (A) 2 min; (B) 7 min. The broken line shows the second scan.

TABLE 1

Comparison of the height of zinc peaks in samples with and without cysteine by d.p.a.s.v. (Flow rate 1.8 ml min⁻¹, sample volume 500 μ l, plating time 60 s, plating potential -1.35 V)

Zn(II) ($\times 10^{-12}$ mol)	L-Cysteine ($\times 10^{-9}$ mol)	Zn(II) I_p^a (μ A)	$I_p/\text{Zn(II)}$ ($10^9 \mu\text{A mol}^{-1}$)
15	—	0.125	8.33
15	42.5	0.110	7.33
25	—	0.240	9.60
25	41.5	0.210	8.40
150	—	1.42	9.47
150	41.5	1.25	8.33

^aEach value is a mean of 10 determinations. Standard deviation 6% at 30 mM and 4% at higher zinc concentrations.

stripping. In contrast, the much thicker film obtained when stripping is done into a solution containing cysteine (Fig. 3) clearly decreases the rate of zinc transport. The medium exchange thus improves the prospects for quantitative d.p.a.s.v. in matrices containing interfering compounds. The detection limit of zinc is also improved partly because of a decrease in background current and partly because of the better curve shape at very low zinc levels.

DISCUSSION

The polarographic reversibility of zinc depends on the buffer constituents and the pH. The medium exchange makes it possible to strip into an acetate buffer in which the zinc wave is unusually well-behaved. The evaluation can be made from voltammograms recorded in the same medium irrespective of the different buffer ions used for the samples.

Film-forming substances interfere seriously with all voltammetric methods. A film formed during plating in a sample containing cysteine remains on the drop after medium exchange and is only slowly desorbed. The film does not have a very significant effect on the rate of zinc transport into or out of the mercury. Tentative experiments with another film-forming substance, Triton X, indicate that in this case the transport of metal ions is indeed affected by the presence of the film. Any definite conclusion should therefore await a more extensive investigation.

The electrochemistry of cysteine is very complex, as evidenced by two recent studies [18, 19]. Experiments in a flow-through cell with the f.i.a. technique seem to provide more information than was available from the methods used in those studies. Medium exchange experiments might be useful in mechanistic studies of film-forming substances.

From the analytical point of view, a combination of flow-through d.p.a.s.v. with f.i.a. offers several advantages. The most obvious is the saving of time compared to traditional d.p.a.s.v. The increase in sample throughput may be very substantial if only a short plating time is required. A very thorough

deaeration is necessary for normal d.p.a.s.v. of trace metals. With the present technique a large reservoir of carrier electrolyte has to be kept oxygen-free but the samples themselves need only to be deaerated very briefly. A flow-through arrangement is easily automated and a computerized version of the above technique is currently being developed. The advantages of flow-through methods become more apparent as additional steps are added. A flow-through dialyzer combines well with the f.i.a. technique [20] and can thus be added without any decrease in sample throughput.

Because of the general occurrence of zinc in laboratory air, in chemicals and on glass, decontamination is more difficult than for any other heavy metal. The flow-through f.i.a.—d.p.a.s.v. system is closed, and so can easily be kept clean all the time. The amount of glassware used and the handling of the sample can both be reduced compared to batch-wise d.p.s.a.v.

This work was supported by grants from the Swedish Natural Science Research Council.

REFERENCES

- 1 See, e.g., P. T. Kissinger, *Anal. Chem.*, **49** (1977) 447 A.
- 2 R. J. Rucki, *Talanta*, **27** (1980) 147.
- 3 J. S. Burmicz, in W. F. Smyth (Ed.), *Electroanalysis in Hygiene, Environmental, Clinical and Pharmaceutical Chemistry*, Amsterdam, 1980, p. 309.
- 4 W. R. Seitz, R. Jones, L. N. Klatt and W. D. Mason, *Anal. Chem.*, **45** (1973) 840.
- 5 S. H. Lieberman and A. Zirino, *Anal. Chem.*, **46** (1974) 20.
- 6 J. Wang and M. Ariel, *J. Electroanal. Chem.*, **83** (1977) 217.
- 7 J. Wang and M. Ariel, *Anal. Chim. Acta*, **99** (1978) 89.
- 8 J. Wang and M. Ariel, *Anal. Chim. Acta*, **101** (1978) 1.
- 9 B. Lazar and S. Ben-Yaakov, *J. Electroanal. Chem.*, **108** (1980) 143.
- 10 A. Ivaska and W. F. Smyth, *Anal. Chim. Acta*, **114** (1980) 283.
- 11 G. Koster and M. Ariel, *J. Electroanal. Chem.*, **33** (1971) 339.
- 12 A. M. Bond, H. A. Hudson and P. A. van den Bosh, *Anal. Chim. Acta*, **127** (1981) 121.
- 13 P. Maitoza and D. C. Johnson, *Anal. Chim. Acta*, **118** (1980) 233.
- 14 E. O. Martins and G. Johansson, *Anal. Chim. Acta*, **116** (1980) 53.
- 15 S. L. Phillips and I. Shain, *Anal. Chem.*, **34** (1962) 262.
- 16 J. Růžička and E. H. Hansen, *Anal. Chim. Acta*, **114** (1980) 19.
- 17 H. B. Hanekamp and H. J. van Nieuwkerk, *Anal. Chim. Acta*, **121** (1980) 13.
- 18 M. T. Stankovich and A. J. Bard, *J. Electroanal. Chem.*, **75** (1977) 487.
- 19 P. Bianco and J. Haladjian, *Electrochim. Acta*, **25** (1980) 1317.
- 20 L. Gorton and L. Ögren, *Anal. Chim. Acta*, **130** (1981) 45.

SOLUTE TRANSFER IN ON-LINE ANALYTICAL FLOW-THROUGH DIALYZERS

Bernhardsson, Bo

Department of Mathematics, University of Lund, P.O.Box 725,
S-220 07 Lund, Sweden

Martins, Eduardo and Johansson, Gillis^{*}

Department of Analytical Chemistry, University of Lund, P.O.Box 740,
S-220 07 Lund, Sweden

Flow-through dialyzers are extremely useful components in analytical flow systems, in particular in manifolds for fast flow injection analysis (f.i.a.). A sample stream or a sample plug in a carrier stream is pumped through a narrow channel on one side of a membrane while water or a buffer is pumped on the other side. Ions or small molecules will readily pass into the detector channel but particles, cells and macromolecules will be retained on the sample side. Such an on-line separation may be able to remove malfunctions or interferences on the detector side due to e.g. clogging, side reactions, growth in beds of immobilized enzymes, or light scattering and absorption in optical cells.

Dialysis has been little used in analytical chemistry because it has been thought to be a slow and not very well defined process. This may be true for equilibrium dialysis or batch-wise operation but not for linear dialysis in a flow system. Some recent applications in f.i.a.-systems show that 45-70 samples per hour can be processed and that the results are highly reproducible [1,2].

The rate of solute transfer between the sample and the detector channels in a flow-through dialyzer depends on the membrane characteristics and on the transverse transport from the bulk of the solution to the membrane surface. The dialyzers can be designed by trial and error and the best one, as judged by the fractional solute transfer, can be evaluated more in detail before it is incorporated into a system. Such a procedure may be rapid and quite successful but a more thorough understanding will probably allow a better optimization and give more confidence in the technique. By comparing experiment with theory it should be possible to separate the effects of geometry and membrane characteristics.

Van der Linden [2] derived a model for gas diffusion in an analytical parallel plate dialyzer by using a tank-in-series model. Each tank is assumed to be mixed and to exchange material with a tank on the other side of the membrane in a time-dependent transfer step. It was proven experimentally that the derived equations predicted the flow dependence correctly. Cooney et. al. [3,4] derived equations which described the mass transfer across the membranes in semi-infinite parallel-plate dialyzers. A special feature of the solution was that two sets of convective diffusion equations, representing the blood and dialysate sides respectively, were coupled through the membrane transfer equations. The theory was developed for artificial kidney dialyzers in which a blood channel is surrounded by a membrane and a dialysate or water channel on each side. There is thus a symmetry around the blood channel which has no correspondence for the sample channel in an analytical dialyzer.

A great number of papers are available on the mass transfer in dialyzers for artificial kidneys [5] or for industrial dialysis, but we were not able to find theories which are directly applicable to analytical dialyzers. One reason is that the concentration on the dialysate side often is assumed to be zero. The approach used by Cooney et.al is attractive because only fundamental constants are needed for Newtonian fluids in contrast to most other derivations which employ a number of engineering notations, and it has therefore been taken as a starting-point for further work in this paper.

THEORY

Two numerical solutions will be derived, the first assumes plug-flows and the second parabolic flows in both channels. The models are compared with experiment and also with the theory derived by van der Linden [2]. The treatment is restricted to coo-flow as it usually results in smaller differential pressure across the membrane. Another reason is that the dispersion in a long dialyzer will be much smaller with coo-flow than with counter-flow.

The models assume two parallel channels of infinite width separated by a dialysis membrane (Fig. 1.). Sample enters from the left and material is transferred from the upper channel down into the detector channel through the membrane. Transport in the y-direction takes place only by liquid flow and in the transverse x-direction only by Fickian molecular diffusion. Since the width is infinite there will be no transport in the z-direction. The velocity profiles are assumed to be fully developed at the entrance ($y = 0$) and the concentrations at $y = 0$ are C_s^0 and C_d^0 respectively. The subscripts s and d are used to denote quantities in the sample and detector channels, but the subscript i is used instead of s and d to save space, whenever the equations have the same forms for both streams. The convective diffusion equations at steady-state are

$$v_i(x) \frac{\partial c_i}{\partial y} = D_i \frac{\partial^2 c_i}{\partial x^2} \quad (1)$$

with the boundary conditions

$$c_i = C_i^0 \text{ at } y = 0 \quad (2)$$

$$\frac{\partial c_i}{\partial x} = 0 \text{ at } x = -a_i/2 \quad (3)$$

Eqn. (3) states that the walls are impermeable. The permeation is assumed to be proportional to the concentration differences between the two sides of the membrane

$$\pm D_i \frac{\partial c_i}{\partial x}(a_i/2, y) = P[c_s(a_s/2, y) - c_d(a_d/2, y)] \quad (4)$$

where P is the permeability ($m s^{-1}$). The permeability has also been defined as the ratio of the diffusion coefficient in the membrane phase divided by the thickness ($m s^{-1}$), which is functionally equivalent with the definition used in Eqn. (4). A third definition has a different meaning, namely the partition coefficient times the diffusion coefficient in the membrane ($m^2 s^{-1}$). Many papers use the Sherwood number, which is the permeability, as in Eqn. (4), times the half channel height divided by the diffusion coefficient in the solution (dimensionless).

The convective diffusion in the two channels are linked through equal-flux boundary conditions at the membrane surfaces as expressed by Eqn. (4). The type of solution depends on the form of $v_i(x)$ in Eqn. (1) and the treatment will therefore be subdivided into two sections covering plug and parabolic flows respectively.

I. Plug flows in both channels

The plug-flow condition can be stated mathematically by

$$v_i(x) = v_i \quad (5)$$

i.e. the linear velocity is the same everywhere within the channels. The solutions are found by Fourier series expansions after the introduction of the reduced variables

$$\begin{aligned} c_i^* &= (c_i - c_d^0) / (c_s^0 - c_d^0) \\ x_i^* &= x_i / a_i \quad K_i^2 = v_i a_i^2 / D_i \end{aligned} \quad (6)$$

$$c^*(x^*, y) = \sum_0^{\infty} B_n e^{-\lambda_n^2 y} F_n(x^*) \quad (7)$$

where λ_n and F_n are the eigenvalues and eigenfunctions of

$$F_i'' + \lambda^2 K_i^2 F_i = 0 \quad (8)$$

$$F_i'(-\frac{1}{2}) = 0 \quad (9)$$

$$-\frac{D_i}{Pa_i} F_i'(\frac{1}{2}) = F_s(\frac{1}{2}) - F_d(\frac{1}{2}) \quad (10)$$

The constants B_n in Eqns.(7) are determined by application of the inlet conditions at $y = 0$ which leads to

$$B_n = \frac{\frac{D_s}{a_s} \int_{-\frac{1}{2}}^{\frac{1}{2}} F_{ns}(x^*) K_s^2 dx_s^*}{\frac{D_s}{a_s} \int_{-\frac{1}{2}}^{\frac{1}{2}} F_{ns}^2(x^*) K_s^2 dx_s^* + \frac{D_d}{a_d} \int_{-\frac{1}{2}}^{\frac{1}{2}} F_{nd}^2(x^*) K_d^2 dx_d^*} \quad (11)$$

where s and d stands for integration over the sample and detector channels respectively.

The equations form a Stourm-Liouville [6] system and if it is solved the results will be

$$\lambda_0 = 0$$

$$\lambda_n = P \left[\frac{a_d}{D_d K_d} \cot(K_d \lambda_n) + \frac{a_s}{D_s K_s} \cot(K_s \lambda_n) \right] \quad (12)$$

and

$$F_0 \equiv 1$$

$$F_{ni} = g_{ni} \cos(\lambda_n K_i (x_i^* + \frac{1}{2})) \quad (13)$$

where

$$g_{ni} = \pm 1/(\sqrt{D_i v_i} \sin(K_i \lambda_n))$$

λ_n in Eqn. (12) has to be found iteratively by a recursive algorithm. When the eigenvalues λ_n are known it is possible to calculate the eigenfunctions and after that the corresponding constants B_n can be found

$$B_0 = \frac{a_s v_s}{a_s v_s + a_d v_d} \quad (14)$$

$$B_n = \frac{2 g_{ns} v_s a_s \frac{\sin(\lambda_n K_s)}{\lambda_n K_s}}{g_{ns}^2 v_s a_s \left[1 + \frac{\sin(2\lambda_n K_s)}{2\lambda_n K_s} \right] + g_{nd}^2 v_d a_d \left[1 + \frac{\sin(2\lambda_n K_d)}{2\lambda_n K_d} \right]} \quad (15)$$

The mixing cup concentrations are mean values within a volume limited by the lengths y_i and $y_i + \Delta y$

$$C_{\text{mix}_S}(y) = \int_{-\frac{1}{2}}^{\frac{1}{2}} C_S(x_S^*, y) dx_S^* = B_0 + \sum_1^{\infty} B_n e^{-\lambda^2 n y} g_{ns} \frac{\sin(\lambda_n K_S)}{\lambda_n K_S} \quad (16)$$

$$C_{\text{mix}_D}(y) = \int_{-\frac{1}{2}}^{\frac{1}{2}} C_D(x_D^*, y) dx_D^* = B_0 + \sum_1^{\infty} B_n e^{-\lambda^2 n y} g_{nd} \frac{\sin(\lambda_n K_D)}{\lambda_n K_D} \quad (17)$$

The expressions consist of one term, B_0 , which is reached asymptotically at large values of y , i.e. a point far downstream in a long dialyzer. The constant B_0 , see Eqn.(14), equals the weighted solute distribution ratio at equilibrium. Ten terms of the transient part of Eqns. (16) and (17) are normally sufficient for three-figures accuracy.

The difference approximation method described below can also be applied to plug-flow if Eqn.(5) is used instead of Eqn.(18). The solutions obtained with the two methods agree within three decimals (with 40 boxes and 1 000 intervals).

II. Parabolic flows in both channels

The velocity profiles for a two dimensional laminar flow regime are approximately given by

$$v_i(x) = v_{i,\text{max}} \left(1 - \left(\frac{x_i}{a_i/2}\right)^2\right) \quad (18)$$

Eqns. (18), (1), (2), (3) and (4) together constitute a system which can be solved numerically by subdividing each channel into boxes (normally 40), as shown in Fig. 2. The concentrations are C_i^0 in all boxes at the beginning and changes with time as the boxes are equilibrated according to the equations, see the Appendix. The procedure used to find the concentration in a forward box from three

existing boxes is shown by the arrows in Fig. 2. The procedure is repeated until the length of the dialyzer has been covered. The respective flow-rate weighted mixing-cup concentrations at a number of y -values are calculated by taking the mean concentrations of vertical rows of boxes. Around 1000 t:s were used for a dialyzer length of 500 mm and the computing time then became 2 - 15 min with a Pascal program in a VAX-11/780 computer.

The mathematical method used by Cooney et.al. [3,4] depends on a symmetry in the blood channel. The analytical dialyzers have one impermeable wall and the concentration profiles will therefore be unsymmetrical and the resulting expressions will contain both even and odd Kummer functions [6]. Numerical solutions for a laminar flow regime will therefore become very complex and require long computer times. The difference approximation method described above is therefore to be preferred, both because of its simplicity, and because it requires less computational power.

EXPERIMENTAL

Four different parallel-plate dialyzers, all made from perspex halves clamped together with the membrane in between, were used. The two analytical dialyzers are shown in Fig. 3. The Tecator AB dialyzer had a 500 mm long rectangular groove, 1.5 mm wide and 0.091 mm deep, which was folded back and forth in smooth curves. A home-made dialyzer had sharp bends where the grooves fold and an O-ring was inserted for additional sealing. The channels were a little deeper ($a_s=0.20$ and $a_d=0.17$ mm), but the width was the same and the length slightly less (475 mm). Two dialyzers with short straight channels and small exposed membrane areas were also used, but they are not described in detail in this paper. The depths of the channels were measured with a microscope for reflected light.

Either a 1×10^{-4} or a 5×10^{-5} M zinc nitrate solution in a 0.15 M sodium acetate buffer, pH 4.70, was used as sample solution when the flow dependence was studied. The detector channel contained an identical buffer except that the zinc(II) was excluded. A larger range of zinc(II)-solutions was used when the linearity was tested.

The solutions were pumped continuously through the dialyzer channels with a peristaltic pump. The zinc(II) concentration was measured at steady state by anodic stripping voltammetry in a flow-through cell as described elsewhere [7].

Cellulose acetate dialysis membranes (Elkey Products Inc., Worcester, Mass., Cat.no. DM-C-2-157-0144) with a thickness of 0.025 mm were used throughout. The membranes were washed and allowed to swell in water or 0.15 M NaCl for at least one day before use. Fresh membranes were taken every few days. The permeation did not change noticeably during this time and it was not affected by the differences in the swelling solutions.

RESULTS AND DISCUSSION

Theory

The theoretical models, which have been derived above, can be used to calculate the concentration in the detector channel for dialyzers of known dimensions if the diffusion coefficient and the permeability are available. The former is readily available for zinc(II) ions in aqueous solution [8], and the latter was determined experimentally as will be discussed below. Fig. 4 shows the predicted variation in the output concentration when the flow rate was varied. The maximum value is reached at equilibrium and it is 0.500 for equal flows in the two channels, assuming a steady-state input concentration of 1.000.

The mixed-cup model described by van der Linden [2] was rewritten for steady-state conditions and its predictions are also shown in Fig. 4. The diffusion coefficient is not used in this model since a perfect mixing is assumed to take place within each tank, see the flow pattern in Fig. 2 C. For the same reason no boundary layer is present and the model does not differentiate between corrected and uncorrected permeabilities.

As expected, the mixed-cup model predicts the highest output concentration because it assumes a perfect transverse mass transfer within each channel. The next highest output concentration is predicted by the plug-flow model, although the difference between it and the laminar-flow model is very small, in particular at low flow rates. The layers adjacent to the membrane surface move much faster in the y-direction in the plug-flow model and more substance should therefore become available in the boundary layers than if a laminar-flow regime is assumed. All three models predict that equilibrium is reached below 0.1 ml min^{-1} .

The predictions of the mixed-cup model is not affected at all by the changed channel dimensions as can be seen from Table 1. The other two predict correctly a decreased output concentration for increased channel heights. It can be seen that, as expected, the differences between the plug-flow and laminar-flow models increase as the channel height increases, because the latter can account for the unsymmetrical concentration distributions in the channels in a more elaborate way. From a practical point of view the differences between the two models derived in this paper are small enough to be negligible. The plug-flow requires a more elaborate program but when it is in the computer it makes the calculations much faster. With a Basic-program in a personal computer the results were obtained in less than half a minute.

An infinite plane in the z-direction has been assumed in the derivation, whereas the channels are of limited width (1.5 mm) in the actual calculations. The problem has been considered by several researchers, for a discussion, see ref. [5] and references cited therein, and it appears that significant deviations between finite and infinite width models start at a height to width ratio of about 0.1, and may, depending on the flow rate, have the same effect as up to a 30% decrease in channel width at a ratio of 0.5. Neglection of the boundary effects at the sides of the channels will thus result in too high values at channel heights above 0.2 mm in Table 1.

The driving force for membrane permeation depends only on the concentration difference across the membrane, and the flux should

therefore be independent of the flow rate if the depletion in the boundary layers are negligible. Table 2 shows the calculated, normalized mean concentrations in the boxes on either side of a membrane for two different flow rates with a 100-boxes parabolic flow model. It can be seen that the concentration in box N on the sample side has decreased from 1.000 (at $y=0$) to 0.700 at the end of the channel and that a corresponding increase has taken place in the detector channel. The mean concentrations are 0.991 and 0.0088 respectively but the concentrations in the middle of the channels are still 1.000 and 0.000 (The Table has been shortened because the profiles in the channels form mirror images). The values in the N and N+1 boxes changed very little if a finer subdivision ($N = 200$) was made. The conclusion to be drawn is that it is necessary to correct for boundary layer depletion in order to calculate a true permeability even when the overall depletion is very small.

So far it has tacitly been assumed that the flow profiles are fully developed at the entrance of the dialyzer. The entrance length, i.e. the distance at which the above condition holds with say 1% accuracy is fairly short for channels of infinite width and low flow rates (fractions of mm for 0.1 ml min^{-1}) but according to theory it should increase significantly as the flow rate and height to width ratio increase [9,10]. Two short dialyzers were therefore made, one with the dimensions given in the head of Table 2 and one with long channels before and after the section with the membrane. Physically it was realized by making a 125 mm long straight parallel plate dialyzer with a teflon tape covering the membrane on the sample side all the way except for a rectangular opening in the middle. The latter cell was used for experimental determination of the permeability.

Comparison with experiments

The concentration of zinc(II) in the detector channel was measured voltammetrically at steady-state conditions and it was found to be strictly proportional to the zinc(II) concentration in the sample

channel when everything else was kept constant. Normalized output concentrations were used throughout and they were computed by dividing the output by the input concentrations (C_d/C_s).

Separation of intra-membrane transport from convective or diffusional transport has been the subject of many theoretical and experimental studies starting with those of Kaufman and Leonard [11]. It is generally accepted that a separation of the effects can be made through a Wilson plot, i.e. the inverse apparent permeability is plotted versus the inverse stirring rate or fluid velocity raised to the power of an empirical factor. The plots yield straight lines with different slopes for laminar and turbulent flows [12]. Extrapolation to zero (infinite liquid flow) gives the corrected membrane permeability. Wilson plots for the dialysis of zinc(II) through the short cell give permeabilities of 7.3 and $8.5 \times 10^{-6} \text{ m s}^{-1}$ with the exponents 0.8 and 0.9 respectively.

The flux can be calculated from the measured concentration in the detector channel and the flow rate and it should therefore be possible to calculate a true permeability directly if the true concentrations are known. The flux through a membrane is given by

$$J = P(C_s^N - C_d^{N+1}) \quad (19)$$

if the difference in osmotic and hydrostatic pressures can be neglected. J is the solute flux per unit membrane area ($\text{moles m}^{-2} \text{ s}^{-1}$) and P is the permeability (m s^{-1}).

The concentration profiles can be taken from the derived model, compare Table 2, and a logarithmic mean concentration be inserted on each side in Eqn.(19). When this was done it was found that the calculated permeabilities depended on the flow rate. This indicates that one or more of the assumptions were violated in practice.

The mean value of the permeabilities above 0.5 ml min^{-1} was $7.74 \times 10^{-6} \text{ m s}^{-1}$ which agrees well with the results from the Wilson plots. The differences between the permeabilities determined in the two short dialyzers were within the experimental errors. A preceeding

section to get fully developed flow profiles therefore seems to be of little value under these conditions. A study of other experimental parameters is needed and the given value of the permeability should be regarded as tentative.

The zinc(II) concentration in the output from the detector channel of the Tecator 500 mm dialyzer was measured when the flow rates in the sample and detector channels were equal (Fig.4). An increasing fraction of solute is transferred to the detector channel as the flows decrease until the equilibrium value, 0.5, is reached. No significance should at this stage be given to the fact that the experimentally found curves are lower than the calculated since the position depends on the value of permeability. Recalculations with other values of the permeability can give better fits. There is no significant difference in this respect between the two large dialyzers.

Fig. 4 suggests that the dialyzers behave according to the assumptions made in the theoretical derivations. This is not true, however, when unequal flows are used in the sample and detector channels. The equilibrium value may be reached at lower or higher flow rates than predicted by theory and the equilibrium value may differ from that derived from the ratio of the flows. It should also be recalled that the depletion-corrected fluxes were dependent on flow rate.

Two causes of deviation between experiment and theory come to mind, namely differences in hydrostatic pressures on the two sides and membrane bulging. It was found that the flux from the sample to the detector channel increased significantly if the waste receiver for the sample channel was put on a higher shelf, a membrane bulging effect was also indicated by a changed flux after a flow purge through one channel. The results in Fig. 4 are obtained with a membrane which has never been subjected to excessive or unequal flows, if that had been the case the experimental curve might have deviated substantially from the shown curve, especially at very low flow rates.

The experimental behavior of the flow-through dialyzers seems to follow the theoretical mass transfer behavior only if some precautions are taken. i) The hydrostatic pressure should be equal in the two channels and this requires that the flow rates are equal (if the channel dimensions are equal) and that the in- and outlet pressures are equal ii) Procedures that could result in membrane bulging should be avoided. The effect of bulging might be less if the channel heights are increased but at the expense of the transfer of solute, compare Table 1. Larger channels will also increase the dispersion if the dialyzer is used in a f.i.a.-system but this can be partially off-set by filling the channels with glass-beads [2,13,14]. If this can be done very precisely it might even restrict the movement of the membrane. The mixed-cup model should be a better representation of the behavior of filled channels than either of the other theories. iii) The flow-rate should be high enough to reach a more leveled-out part of the flow-dependence curve (Fig. 4), i.e. at most 25% of solute should be transferred (with equal dimensions and equal flows). The reason is that the concentration ratio varies less with flow rate and thus with membrane bulging.

Peristaltic pumps give a pulsed flow whereas the theory assumes a steady-state flow. With the experimental conditions used in this work the effect on the mass liquid transfer pattern should be negligible [15], but it may be of importance if the pulsations are out of steps both because of differences in hydrostatic pressures and membrane bulging. The folds and grooves may result in some secondary flow [16] but it should be of less importance with the small height to width ratio of the analytical dialyzers.

The authors thank Dr G. Sparr, Department of Mathematics, for valuable help with the mathematical solutions. Financial support by the Swedish Natural Research Council is acknowledged.

APPENDIX

Eqn. (1) is rewritten with differentials

$$\frac{C(x, y + \Delta y) - C(x, y)}{\Delta y} = \frac{1}{K_i^2(x)} \cdot \frac{C(x - \Delta x, y) - 2C(x, y) + C(x + \Delta x, y)}{(\Delta x)^2} \quad (20)$$

where $K_i^2(x) = v_i(x) a_i^2 / D_i$
with the boundary and initial conditions

$$C_1 = C_2, \quad C_{2N} = C_{2N-1} \quad (21)$$

$$C_{(1 \text{ to } N)} = 1, \quad C_{(N+1 \text{ to } 2N)} = 0 \text{ for } y = 0$$

See Fig. 2 for box designations. The membrane equations Eqn.(4) become

$$D_d(C_{N+2} - C_{N+1}) / \Delta x = D_s(C_N - C_{N-1}) / \Delta x = P(C_{N+1} - C_N) \quad (22)$$

The computations involve finding the concentration in a cell at $x, y + \Delta y$ from the three previous cells $x - \Delta x, y$; x, y ; $x + \Delta x, y$ by using Eqn.(20). The computations begin by calculating the concentration of box 2 at $y + \Delta y$ (Box 1 gets its concentration from Eqn.21) and the procedure is repeated for each box until $N-1$. A similar procedure is then used for the boxes $2N$ to $N+2$. The concentrations in boxes N and $N+1$ are finally obtained by Eqn.(22). The procedure is continued until the sum of Δy equals the length of the dialyzer. In order to obtain stable solutions Δy has to be selected so that [6]

$$0 < \Delta y < 0.5 \min K_i^2(x) (\Delta x)^2 \quad (23)$$

REFERENCES

1. L. Gorton and L. Ögren, *Anal. Chim. Acta* 130(1981)45.
2. W.E. van der Linden, *Anal. Chim. Acta* 151(1983)359.
3. D.O. Cooney, E.J. Davis and S-S Kim, *Chem. Eng. J.*, 8(1974)213.
4. D.O. Cooney, S-S Kim and E.J. Davis, *Chem. Eng. Sci.* 29(1974)1731.
5. J.M. Kooijman, *Chem. Eng. Sci.*, 28(1973)1149.
6. M. Abramowitz and I.D. Stegun, *Handbook of Mathematical Functions*, N.B.S., Washington, 1964.
7. E.O. Martins and G. Johansson, *Anal. Chim. Acta* 140(1982)29.
8. M. Duflo-Plissonnier, *Radiochem. Radioanal. Lett.*, 8(1971)277.
9. L. Grimsrud and A.L. Babb, *Chem. Eng. Progr. Symp. Ser.* 66, 62(1966)20.
10. R.B. Bird, W.E. Stewart and E.N. Lightfoot, *Transport Phenomena*, Wiley & Sons, N.Y. 1960.
11. T.G. Kaufmann and E.F. Leonard, *AIChE J.* 14(1968)110, 421.
12. K.A. Smith, C.K. Colton, E.W. Merrill and L.B. Evans, *Chem. Eng. Progr. Symp. Ser.* 84, 64(1968)45.
13. J.H.M. van der Berg, R.S. Deelder and H.G.M. Egberink, *Anal. Chim. Acta* 114(1980)91.
14. J.M. Reijn, Thesis, Amsterdam University, 1983.
15. J.R. Womersley, *J. Physiol.*, 127(1955)553.
16. R. Tijssen, Thesis, Delft University, 1979.

Table 1

Normalized output concentrations in the detector channel in a dialyzer with equal flow rates, channel heights, lengths = 500 mm, and widths = 1.5 mm. $P = 7.74 \cdot 10^{-6} \text{ m s}^{-1}$, $D = 7.4 \cdot 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

Channel heights mm	Flow rate, ml min ⁻¹				
	0.10	0.20	0.50	1.0	2.0
0.091					
Mixed-cup	0.500	0.485	0.376	0.251	0.147
Plug	0.492	0.438	0.288	0.180	0.106
Laminar	0.492	0.436	0.283	0.174	0.100
0.20					
Mixed-cup	0.500	0.485	0.376	0.251	0.147
Plug	0.469	0.381	0.231	0.146	0.088
Laminar	0.463	0.366	0.220	0.133	0.078
0.5					
Mixed-cup	0.500	0.485	0.376	0.251	0.147
Plug	0.389	0.280	0.165	0.107	0.068
Laminar	0.382	0.266	0.146	0.090	0.054
1.0					
Mixed-cup	0.500	0.485	0.376	0.251	0.147
Plug	0.300	0.208	0.124	0.082	0.054
Laminar	0.285	0.187	0.102	0.063	0.039

Table 2

Concentrations in the boxes surrounding a membrane in the small dialysis cell. The calculation was made with 100 boxes on each side, $C_s^0 = 1.000$, $C_d^0 = 0.000$, $D = 7.4 \cdot 10^{-10} \text{ m}^2 \text{ s}^{-1} [8]$, $P_{\text{init}} = 8.5 \cdot 10^{-6} \text{ m s}^{-1}$, $Q_s = Q_d$, $a_s = a_d = 0.5 \text{ mm}$, y is the distance in the length direction (mm).

Box number	Concentrations			
	0.2 ml min ⁻¹		1.2 ml min ⁻¹	
	y = 2.3	y = 4.6	y = 2.3	y = 4.6
Sample channel				
50	1.000	1.000	1.000	1.000
N - 3	0.809	0.767	0.910	0.873
N - 2	0.784	0.746	0.881	0.845
N - 1	0.760	0.724	0.849	0.816
N	0.735	0.703	0.816	0.785
Detector channel				
N + 1	0.265	0.297	0.184	0.215
Mean conc.	0.0050	0.0088	0.0011	0.0020

Summary

The mass transfer in infinite parallel plate dialyzers with coo-flow between sample and detector streams is discussed for three different theoretical models. Analytical solutions with coupled diffusion and membrane transfer equations were obtained for plug-flow in both channels. The finite difference approximation method was used to obtain numerical solutions for a laminar flow regime. Results obtained with a mixed-cup model under steady-state conditions were also included. With the dimensions typical for analytical dialyzers there were only small differences between the laminar and the plug-flow models. The mixed-cup model predicted higher fluxes through the membrane than the other two models, in particular when the channel heights were increased. The theoretical results were compared with experiments for dialysis of Zn(II) ions and the flow dependence agreed reasonably well with theory provided that the hydrostatic pressures were equal on both sides, and that stresses which could result in membrane bulging were kept low.

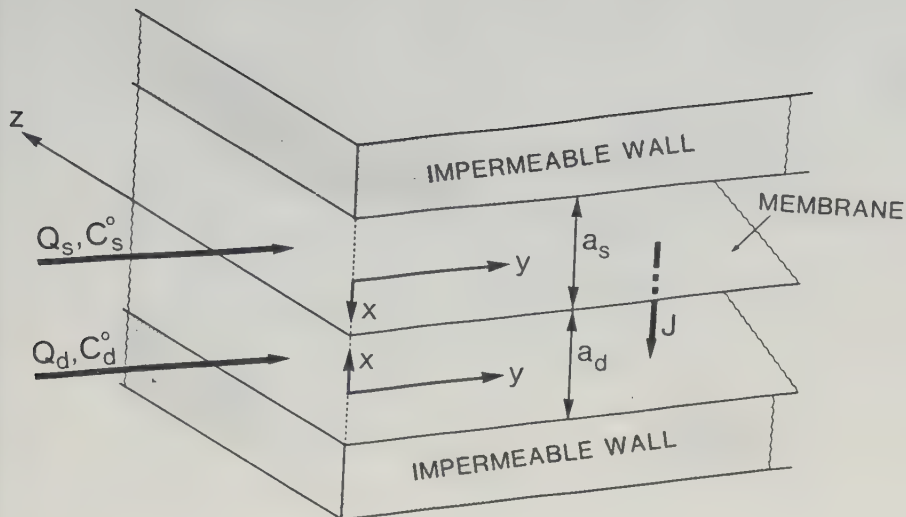


Fig. 1. Model of the theoretical parallel plate dialyzer with symbols and variables.

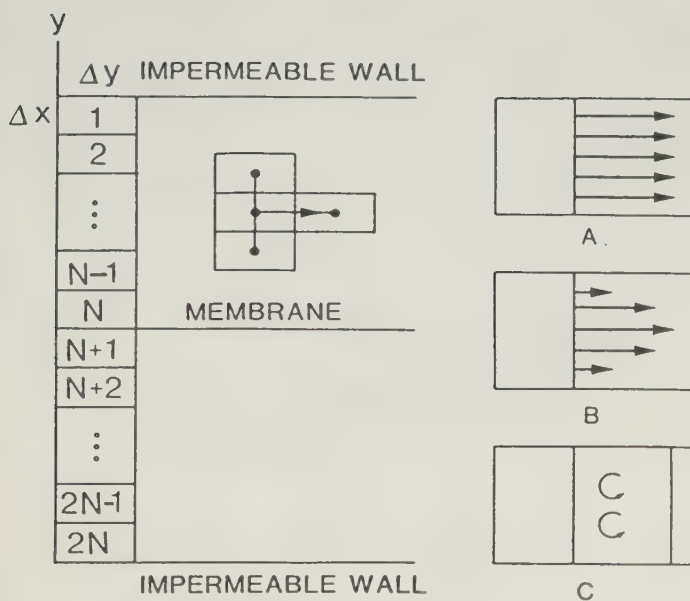


Fig. 2. Box designations used in the finite difference approximation method (left) and the flow pattern assumed in the plug-flow (A), laminar flow (B) and mixed-cup (C) models (right).

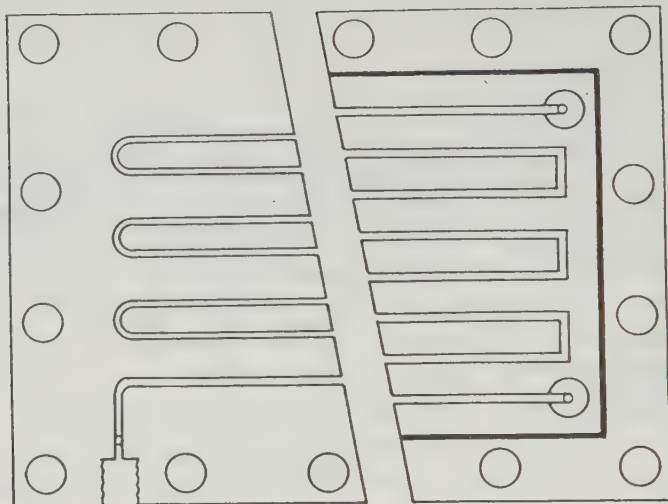


Fig. 3. Top view of the Tecator (left) and home-made (right) dialyzers showing the channel arrangements. The outlets of the latter are directed downwards.

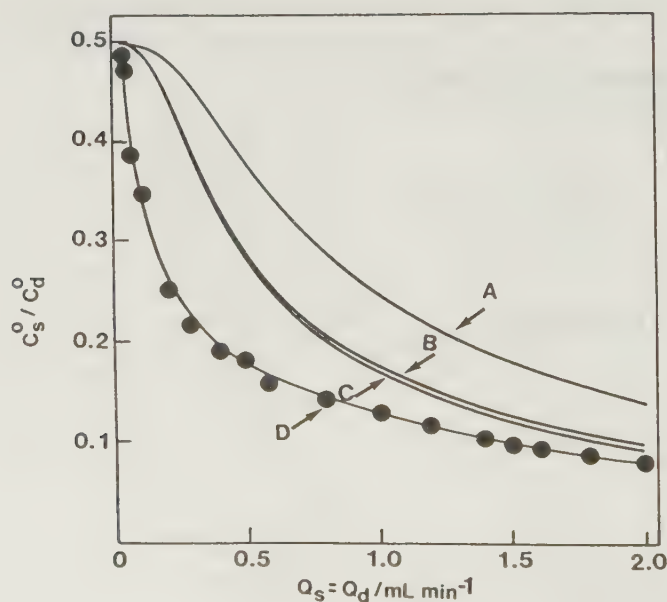


Fig. 4. Flow dependence of the normalized output concentration of a 500 mm long parallel-plate dialyzer, $a_s = a_d = 0.091$ mm, channel widths = 1.5 mm, and equal flows in both channels. $P = 7.74 \cdot 10^{-6}$ m s⁻¹, $D = 7.4 \cdot 10^{-10}$ m² s⁻¹. A, Mixed-cup model, B, Plug-flow model, C, Laminar-flow model, D, Experimental values for Zn(II) ions.

ON-LINE DIALYSIS OF SOME METAL IONS AND METAL ION COMPLEXES

Eduardo Martins, Mats Bengtsson and Gillis Johansson.
Department of Analytical Chemistry, University of Lund,
P.O.Box 124, S-221 00 Lund, Sweden

Summary

The effect of ionic strength, pH and complexing ligands on the dialysis of metal ions, in particular zinc(II), through cellulose acetate membranes, was studied under flow conditions. The dialysis factor, which depends on both the mass transfer and the membrane permeability, was found to be independent of ionic strength in the interval 0,05 to 0,3 M and to increase only slightly with pH between pH 4.6 and 7.0. Some common buffer constituents had no effect, but chloride and calcium ions affected the transfer rates. The rate of transfer of the ligands histidine, NTA and EDTA was of the same order of magnitude as that of the metal ions. The transfer rates of the Ni(II)-EDTA complex was the same as that of a mixture of Ni(II) and EDTA. Generally, addition of the chelating agents decrease the metal ion transfer rates. Partition coefficients between the membrane polymer and the buffers were determined and compared with the dialysis factors. The time scale, the range of variation, the concentration and pH-dependence differed significantly which indicate that the dialysis is unrelated to the polymer uptake or release of metal ions.

INTRODUCTION

Dialysis is basically a separation process for different solutes by means of their unequal transport rates through membranes. The driving force for dialysis is thus the concentration gradient across the membrane. Dialysis has been used for a very long time beginning with the separation of colloids in solution [1]. Today dialysis is an important method in biochemistry for the separation of small solutes from proteins and other macromolecules. Dialysis is also used industrially, in artificial kidneys and recently in analytical flow systems.

A large number of other membrane separation methods are in frequent use, e.g. reverse osmosis, piezodialysis, ultrafiltration, electro-dialysis and electroendosmosis [2]. In reverse osmosis a hydrostatic pressure exceeding the osmotic pressure is applied over the membrane to force a solvent to pass from the high-concentration to the low concentration side. Piezodialysis is the permeation of solutes, by virtue of a pressure difference, in contrast to the solvent flow of reverse osmosis. Ultrafiltration is frequently used as a synonym for reverse osmosis in particular for separation of solutes with molecular dimensions within one order of magnitude of those of the solvent.

Dialysis in an analytical flow system is arranged so that a fraction, say 3-30% of a solute passes from a sample stream containing unwanted components, e.g. particles or macromolecules, to a detector stream containing pure buffer at the entrance of the dialyzer. The fraction is independent of the concentration of solute in the sample stream over very wide ranges for charged as well as uncharged [3-6] species. This fact and the good reproducibility at steady state conditions and in particular in flow injection analysis (f.i.a.) should make the flow dialyzer a very convenient tool in analytical applications. For full confidence in the technique it is, however, necessary to study the effect of changes in a number of factors which may differ between the samples and between the samples and the standards.

The so called dialysis equation states that the flux per unit membrane area, J , is proportional to the concentration difference

$$J = P (C_s - C_d) = p (C_s - C_d) / l \quad (1)$$

where C_s is the solute concentration in the sample and C_d in the detector stream, P is the permeability (ms^{-1}), l the membrane thickness and p the permeability per unit thickness ($\text{m}^2 \text{s}^{-1}$).

The concentrations used in eqn(1) are those just outside the membrane surfaces and they are different from those in the bulk of the solution except at equilibrium. The mass transport in dialysis has been studied by several researchers [7,8] and three different mathematical models which was especially adapted to the analytical dialyzers were recently described [9]. The solute transfer is jointly determined by the solution mass transfer and the membrane permeability, eqn.(1) and it is conveniently expressed by the dialysis factor. It is defined as the output concentration of the detector channel (C_d) normalized by division with the initial sample channel concentration (C_s^0).

The solution mass transfer depends on the integral diffusion coefficient of the solute. There will, however, be a coupling of diffusional transport of the solute and other ions in the solution. Very few data are available on mixed diffusion coefficients and none are available for zinc(II). Changes in the dialysis factors reported in this paper include the effect of diffusional coupling in the solution as well as changes in the membrane permeability.

EXPERIMENTAL

The flow-through dialyzer (Tecator AB) consisted of two planar Perspex halves clamped together with the membrane in between. A rectangular groove, width 1.5 mm, depth 0.091 mm, was cut in an undulating pattern so that the total groove length became 500 mm. A fraction of the solute was thus transferred from one such channel to an identical channel on the other side of the membrane. For equal flow rates in both channels, the fraction became 0.5 at equilibrium,

which occurred at flow rates less than 0.05 ml min^{-1} . The flow rate during actual measurements were 0.5 or 1 ml min^{-1} in both channels.

Cellulose acetate dialysis membranes (Elkey Products Inc., Worcester, Mass., Cat.no. DM-C-2-157-0144) with a thickness of 0.025 mm were used throughout. They were thoroughly washed in water, acetate buffer and water before use. Dialysis factors determined on different occasions, and with different membranes differed less than 6% and a membrane seemed to give constant factors over weeks.

Sorption and desorption of solutes from membranes were determined by the usual method [10], i.e. the membranes were equilibrated for 48 or 72 hours in known volumes of salt solutions and buffer respectively. The decrease or increase in metal ion concentration in the aqueous solutions due to the sorption or to desorption from the membrane was determined by atomic absorption spectroscopy. The equilibration was made in beakers kept in closed vessels containing water to prevent volume changes. The membranes were removed from the metal salt solutions, rinsed with water, and quickly blotted with filter paper and placed in a known volume of buffer for desorption.

Dialysis factors were determined in a flow system consisting of a four-channel peristaltic pump (Gilson, Minipuls 2) with a steady-state flow of sample solution in one channel and buffer in the other. The output of the latter was connected to a flow-through detector with a hanging mercury drop electrode [11] and the metals were determined by differential pulse anodic stripping voltammetry. Separate calibrations were made for each flow rate, pH and buffer composition by pumping solutions directly to the detector. Most measurements were also checked with atomic absorption spectroscopy and it was the sole technique for calcium and magnesium.

The rate of transfer through the membrane of free histidine, NTA and EDTA was determined in a dialysis flow system allowing either the dialysate or the retentate to react with Co(II) and monitoring the absorbance of the coloured complex at 510 nm (LKB-2151 Variable wavelength monitor).

The acetate buffer and the sodium chloride was made from Suprapur salts (Merck AG), the phosphate buffers and the metal salt solutions were made from pro analysi chemicals (Merck AG). The HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane) was of Sigma's regular quality. All buffers were sodium salts. All the solutions were degased with an inert gas (N_2 or Ar) prior to the experiments.

RESULTS AND DISCUSSION

Changes in pH, ionic strength and buffer ions

The effect of sample composition on the dialysis factor for zinc ions was studied by varying the pH of the sample solution while the pH in the detector channel was kept constant (Fig. 1). Acetate, phosphate and HEPES buffers were used in going from the acid to the neutral pH. It can be seen that the dialyzed fraction changes only slightly and monotonically with the increase in pH. Besides zinc ions, also buffer ions and protons will pass through the membrane when the pH or buffer ions are different in the sample and detector channels. Similar results were obtained when the pH on the detector side was changed while that on the sample side was kept constant. The dialysis factors also increased in this case when the pH in the dialysis channel was increased although there was some slight differences in the curve shapes. The curves were namely slightly convex downward in contrast to those in Fig. 1 which are slightly convex upward. The dialysis factors differed at most 5% from those given in Fig. 1.

The co-transport of zinc ions due to transport of buffer ions or protons should therefore be small and the effects are most likely due to changes in protonization of the acetate groups of the membrane itself. The isoelectric point for cellulose acetate is reported to be $pH\ 3.0 \pm 0.2$ [10] and the membrane acquires an excess of negative charge at higher pH, which is expected to facilitate the transport of zinc ions.

The transport rate through a membrane is determined by both the permeability and the mass transfer in the aqueous solutions. Computer modelling with a plug-flow model [11] shows that when the permeabi-

lity is decreased 10% from its experimental value for zinc ions, the dialysis factor will decrease 3.8% at 0.5 ml min^{-1} and 4.9% at 1.0 ml min^{-1} . The effects on the permeability alone should therefore be three times larger than the compounded effects shown in the figure. The dialysis factors will be more dependent on the permeability at high flow rates where the dialyzed fraction is smaller. The reason is of course that the concentration gradient is steeper in the vicinity of the membrane due to less depletion in the sample solution.

The effect on changes in ionic strength is shown in Fig. 2. The composition and pH on the sample and detector sides were equal in each run. The dialysis is practically independent of the ionic strength from $I = 0.06$ to 0.20 M and it is also independent of pH when it is equal on both sides. The membrane properties are thus hardly at all affected by the ionic composition of the solutions in this range.

The transport rate increases significantly, in particular at low pH when the ionic strength decreases below about 50 mM . At very low ionic strengths, i.e. below the millimolar level (not shown) the permeability will increase drastically and become very sensitive to changes in both pH and sample composition. In unbuffered solutions zinc ions will adsorb to the membrane with release of two protons as found by back titration with glass electrode.

Small differences in ionic strength between the channels have little effect in the range $0.06 - 0.20 \text{ M}$ in buffered solution. For ionic strengths larger than, say 1 M , even slight differences between the channels will result in irreproducible dialysis factors.

From an analytical point of view a window is identified in which small changes in the sample conditions have little effect on the permeability. These differences are still more reduced by the leveling-out effect of the mass transport.

Effect of added ligands

Several ions and ligands will affect the rate of transport of metal ions through a membrane. If chloride ions in the acetate buffer mentioned above are replaced by nitrate ions the dialysis factor will increase from 0.165 ± 0.008 to 0.185 ± 0.009 . On the other hand the dialysis factor remained constant when the acetate was replaced by phosphate, HEPES or bicarbonate buffer in the presence of chloride. Decreasing the chloride ion concentration down to 1 mM had no effect as long as the total ionic strength was kept above 50 mM by buffer ions. These observations indicate that the presence of chloride ions affects the membrane permeability in a rather specific way.

Table 1 shows that the dialysis factors for zinc(II) decrease upon addition of chelating ligands in the sample channel or in both the sample and detector channels. The experiment was repeated with 0.5 mM zinc(II) concentrations and the dialysis factor became identical to those given in Table 1. The experiments were also repeated at 5 and 20 times lower ligand concentrations and again the same dialysis factors were obtained. The differences in the dialysis factors shown in Table 1 are thus independent of both the metal and the ligand concentrations.

Table 2 shows the dialysis factors for the ligands themselves (in acetate buffer with chloride ions). It can be seen that the transport rate is of the same order of magnitude as that of the zinc ions. If the dialysis factors are plotted versus the square root of the molecular weight a straight line will be obtained. The slope was in fact the same as that obtained for some carbohydrates in the same dialyzer [12]. The linearity indicates that the difference in transfer rates can be ascribed to changes in diffusion coefficients in the water and in the membrane phases. The histidine should have one negative charge and the other ligands two. Histidine and EDTA have pKs at 6.08 and 6.16 respectively [13,14]. The different charges and the changed protonization seem both to have little effect on the transport rate.

Returning to Table 1 it can be seen that the transport of zinc(II) is slower when NTA and EDTA are present in both channels than when they are present originally only in the sample channel. Since the ligands are in excess in the sample solution and since they are transferred through the membrane (cf Table 2) with almost the same rate as zinc(II) there should be excess of ligand in the detector channel independently of whether it is added to the detector channel carrier or not. The different transfer rate cannot be due to differences in the binding of zinc(II) but might be related to membrane-specific effects. Furthermore it was found that the behaviour was the same for diluted samples and lower ligand concentrations as long as the ligand is in excess. A net transport of NTA and EDTA through the membrane thus seems to increase the transport rate of zinc(II).

Similar experiments were made with cobalt(II) and nickel(II) to see whether the rate of ligand exchange was important (Table 3). The metal chlorides were injected into a flow of buffered ligand which passed directly into the dialyzer. There should thus be little time for equilibration because of the short residence time in the dialyzer. At least for nickel(II) the concentration of metal complex should be negligible, because of the slow rate of complex formation [15,16,17].

Samples were also prepared by mixing nickel(II) and EDTA 72 hours before injection. It can be seen from Table 3 that the dialysis factors are the same in freshly mixed and in aged nickel(II) samples. The diffusing species in the aqueous solutions should be the metal ions in the former case and the metal complexes in the latter. The data thus indicate that nickel(II) can pass the membrane either as metal complexes or as ions or as ion pairs. The striking observation is that the overall dialysis factor is almost the same in both cases. Since the metal complex should have a lower diffusion coefficient in the aqueous solution, this means that it should have a correspondingly higher membrane permeability. The dialysis factor for zinc(II) was the same before and after each experiment. Stopped flow experiments were also made to see whether any metal, metal complex or ligand was retained in the membrane and washed out on a

longer time scale. No such hold-up was observed for any metal or ligand.

A membrane transport inhibition by calcium(II) is known from studies of reverse osmosis [18] and it parallels the calcium blockage of transport in biological membranes. Table 4 shows that calcium ions have a similar effect in dialysis and that the inhibition can be partly reversed by large excess of histidine. If calcium(II) was present in the same concentration in both channels it had no effect on the dialysis factor of zinc. Magnesium(II) was also tested but it showed no transport inhibition whatsoever.

It is not possible to explain the observations quantitatively and not even to indicate a single cause of the decreasing dialysis factors on addition of some ions. The data indicate that all species can pass the membrane, i.e. the metal ions, the ligands and the metal complexes. The last conclusion is supported by the high dialysis factor of the aged nickel-EDTA solution.

The effect of calcium can be explained if it is assumed that the calcium ions use the same transport path or fixed counter-ions as the zinc ions. A net transport of calcium(II) will then retard the zinc(II) transport. When calcium(II) is present in the same concentration on both sides there will be no net transport and the zinc(II) transport will be the same as in the absence of calcium(II).

An analogous explanation may apply to the behavior of the doubly charged ligands (cf Table 1). When the ligand is present on both sides there will be no net transport, but when it is present only on one side it will, due to its negative charge, increase the transport rate of zinc(II). Histidine has only one charge and it will partly be present as a neutral species and it does not increase the net transport of zinc(II).

If the membrane is negatively charged it should reject negative species like the free ligands (Table 2) or the highly negatively charged metal complexes. It was found, however, that the dialysis factors of the ligands themselves decreased with size in the same

manner as the uncharged carbohydrates. The double-charged EDTA complexes of zinc(II) (Table 1) dialyze faster than the single charged NTA complex. The repulsion due to fixed charges in the membrane seems therefore to be negligible.

Partition to the membrane phase

The uptake of metal ions by cellulose acetate membranes has been extensively studied in the field of reverse osmosis [10]. Data were not available for the low concentrations studied in this work and not for many of the ions of interest in analytical dialysis. The previous data were therefore extended using the same experimental methods as described by Heyde, Peters and Anderson [10].

The diffusion of zinc(II) into or out from a package of 10 membranes is shown in Fig. 3. The transport rates are not equal in the two directions which agrees with earlier observations [9]. The diffusion coefficients calculated according to eqn.(39) in Cranck and Park's work [20] became $8,5 \cdot 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ for the desorption and $6,4 \cdot 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ for the sorption, which is the same order of magnitude as reported for other ions [10]. Similar measurements with a single membrane were less precise but paralleled those given in Fig. 3. With a single membrane, saturation was approached after two to three hours.

The partition coefficients for some metal chlorides and nitrates between the membrane and an acetate buffer are given in Table 5. The dialysis factors are also given as well as the membrane permeability. The latter was obtained from Wilson plots [21], i.e. by extrapolation to infinite flow rate. The extrapolation is made in order to obtain a permeability free from solution mass transfer effects.

Table 6 shows the pH-dependence of the partition coefficients and it can be seen that it decreases with pH for Ca(II) and Mg(II) and increases with pH for Zn(II). It should be mentioned that the dialysis factors for Ca(II) and Mg(II) were found to be independent of pH.

Fig. 4 shows the partition coefficient variation with concentration with the data reported by Heyde et al. [10] also shown. Some partition coefficients between membrane and acetate buffers containing histidine are also shown. The values found in this work seem to fit the previous data and the previously given equations (fully drawn lines) fairly well.

A comparison of partition coefficients with dialysis factors shows several important differences and few if any similarities. The partition coefficients are concentration dependent while the dialysis factors are not. The partition coefficients for the different ions range over three and a half power of ten while the dialysis factor vary at most by six times ($\text{Ni}(\text{NO}_3)_2$ and CaCl_2 in Table 5). Changes of the anion produce much larger effects on the partition coefficient than on the dialysis factor. If permeabilities are used in the comparison instead of dialysis factors there was at most a three fold variation between the salts. The time scale for a partition is of the order of hours while that of dialysis is faster than that which can be resolved by a flow injection experiment. No hold up or memory effects or changed dialysis factors with time could be observed.

The conclusion is that there seems to be no correspondence between solute behaviour in membrane diffusion and dialysis. The dialysis seems to be a fast process where the differences between different ions or salts are of the same order as the differences expected for diffusion in aqueous solutions.

A membrane model with two different phases is thus indicated. The slow process involves diffusion into a polymer phase and the partition isotherm of this phase is nonlinear and the equilibrium differs over orders of magnitude between different salts. The other phase should have properties more equal to an aqueous solution [22] and this phase should contain most of the water of the swelled membrane (about 15% w/w). There may be specific interaction for some ions, as indicated by the inhibition of zinc(II) transport by a simultaneous transport of calcium(II), but not by a simultaneous transport of

magnesium(II). This difference may or may not be related to the larger polymer uptake of calcium than of magnesium, compare Fig. 4.

The described behavior of metal ion transport is valid in the presence of excess inert salt or buffer. At low ionic strengths a completely different behavior is indicated and it may possibly be described by the very involved TMS theory [23,24,25].

Relevance for analytical dialysis of metal ions

The dialysis factor determined by steady state measurements and by peak height measurements in flow injection analysis were the same, as were the peak shapes at the outlets of the sample and detector channels.

If the ionic strength and pH are kept within some specified, rather wide limits, the dialysis factors seem to be independent of changes in the sample composition. Generally it should be advantageous to use solutions of the same compositions in both the sample and the detector channels. This is not always possible in practice but some indications of important factors are given by the data presented above. If chloride ions are present in some samples it should be added to the carriers and their concentration does not seem to be important. If calcium(II) is present in the samples it should be balanced by a similar calcium(II) level in the detector channel. A number of other inert ions, exemplified by the buffer ions, seems to be of minor importance for the size of the dialysis factor. Some chelate forming ions decrease the dialysis factor and the effect seem to be independent of both the metal and ligand concentration as long as the latter is in excess. A proper selection of carriers in the two channels should therefore give consistent dialysis factor independent of variations of ligand compositions of the samples. A prediction of the effect of various ligands is not possible at present.

Support from the Swedish Natural Science Research Council and from Perstörpsstiftelsen are acknowledged.

REFERENCES

- 1 T. Graham, *Phil. Trans. Roy. Soc. (London)* 144(1854)177.
- 2 S. Hwang and K. Kammermeyer, *Membranes in Separations*, Wiley, New York, 1975.
- 3 R. Dean, *Chem. Rev.* 41(1947)503.
- 4 L.C. Craig, T.P. King and A. Stracher, *J. Am. Chem. Soc.* 77(1955)6620.
- 5 P.C. Farrel and A.L. Babb, *J. Biomed. Mater. Res.* 7(1973)275.
- 6 K.K. Steward, *Adv. Prot. Chem.* 31(1977)135.
- 7 J.M. Kooijman, *Chem. Eng. Sci.* 28(1973)1149.
- 8 W.E. van der Linden, *Anal. Chim. Acta* 151(1983)359.
- 9 B. Bernhardsson, E. Martins and G. Johansson, submitted to *Anal. Chim. Acta*.
- 10 M.E. Heyde, C.R. Peters and J.E. Anderson, *J. Colloid. Interf. Sci.* 50(1975)467.
- 11 E. Martins and G. Johansson, *Anal. Chim. Acta* 140(1982)29.
- 12 B. Olsson, H. Lundbäck and G. Johansson, submitted to *Anal. Chim. Acta*.
- 13 R.J. Sundberg and R.B. Martin, *Chem. Rev.* 74(1974)471.
- 14 A. Ringbom, *Complexation in Analytical Chemistry*, Interscience, New York, 1963.
- 15 J.C. Cassatt, W.A. Johnson, L.M. Smith and R.G. Wilkins, *J. Am. Chem. Soc.* 94(1972)8399. 18 E. Mitsouyamis and G.D. Saravacos, *Desalination* 21(1977)235.
- 16 J.C. Cassatt and R.G. Wilkins, *J. Am. Chem. Soc.* 90(1968)6045.
- 17 K. Kustin and J. Swineheart, *Progr. Inorg. Chem.* 13(1970)107.
- 18 E. Mitsouyamis and G.D. Saravacos, *Desalination* 21(1977)235.
- 19 G.S. Park in *Diffusion in Polymers* (J. Crank and G.S. Park, eds.), Academic Press, London, 1968, p. 141.
- 20 J. Crank and G.S. Park, eds. *Diffusion of Polymers*, Academic Press, London, 1968.
- 21 T.G. Kaufmann and E.F. Leonard, *AIChE J.* 14(1968)110, 421.
- 22 E. Glueckauf, *Desalination* 18(1976)155.
- 23 T. Teorell, *Proc. Soc. Expl. Biol. Med.* 33(1935)282.
- 24 T. Teorell, *Progr. Biophys.* 3(1953)305.
- 25 K.H. Meyer and J.F. Sievers, *Helv. Chim. Acta* 19(1936)649.

Table 1

Changes in the dialysis factor of zinc ions when various ligands were added. The concentration of zinc ions was 2.5 mM, of NTA and EDTA 0.125 M, and of histidine 0.116 M in acetate buffers also containing 0.15 M NaCl. Sodium acetate was added if necessary to the detector channel to give equal ionic strength on both sides. Flow rate 0.5 ml min⁻¹.

Ligand	Dialysis factor	
	pH 4.67	pH 5.5
-	0.165	0.166
Ligand in sample channel		
Histidine	0.158	0.151
NTA	0.117	0.129
EDTA	0.139	0.129
Ligand in both channels		
Histidine	0.155	0.150
NTA	0.093	0.095
EDTA	0.116	0.096

Table 2

Dialysis factors for histidine, NTA and EDTA (Na-salts).

Sample and receiver channels: NaCl-acetate buffer, total ionic strength 0.15 M, flow rate 0.5 ml min⁻¹.

	histidine	NTA	EDTA
pH 4.7	0.168	0.143	0.135
5.5	0.171	0.147	0.138

Table 3

Dialysis factors for NiCl_2 and CoCl_2 in the presence of histidine, NTA and EDTA (Na-salts) in the sample channel. The concentrations of Ni(II) and Co(II) were 2.5 mM, of NTA and EDTA 0.125 M and of histidine 0.116 M, in acetate buffers, pH 5.5, containing NaCl to a total ionic strength of 0.15 M on both sides.

Ligand	Dialysis factor	
	NiCl_2	CoCl_2
-	0.158	0.170
histidine	0.138	0.150
NTA	0.174	0.071
EDTA	0.140	0.132
EDTA, aged mixture	0.144	0.132

Table 4

Effect of Ca^{2+} and/or histidine on the dialysis of zinc ions. Total zinc concentration $1.5 \cdot 10^{-6}$ M. Flow dialysis. The sample and receiver channels contained NaCl in acetate buffer (50 mM) to a total ionic strength of 0.15 M. Flow rate 0.5 ml min^{-1} .

Conc. of added salts		pH 4.7		pH 5.5	
Ca Cl_2 mM	Histidine-Na mM	% free Zn^{2+}	dialysis factor	% free Zn^{2+}	dialysis factor
-	-	94	0.168	94	0.168
-	0.015	90	0.165	45	0.163
-	0.150	88	0.165	32	0.163
-	1.70	20	0.158	15	0.158
-	2.00	10	0.158	<8	0.158
1.24	-	94	0.168	94	0.168
2.90	-	94	0.160	94	0.160
3.75	-	94	0.135	94	0.134
6.00	-	94	0.136	94	0.134
6.00	0.015	90	0.136	45	0.134
6.00	0.150	88	0.136	32	0.136
6.00	1.70	20	0.158	15	0.158
6.00	2.00	10	0.158	<8	0.158

Table 5

Partition coefficients of some chlorides and nitrates.

a) The membrane/aqueous phase partition coefficient was obtained from adsorption (48H h) of salts, in acetate buffer, pH 4.7, $u=0.15$ M, and desorption (74 h) into 0.15 M acetate buffer, pH 4.7). b) Flow dialysis: Sample and receiver channels containing acetate buffer, 0.15 M, pH 4.7. Flow rate 0.5 ml min^{-1} . c) Permeability corrected for boundary layer effects.

Salt	Conc. mM	n	Partition ^{a)} coefficient	Dialysis ^{b)} factor	Permeability ^{c)} m s^{-1}
CaCl_2	5	10	0.92 ± 0.15	0.302 ± 0.014	16.20 ± 0.73
MgCl_2	2	10	0.081 ± 0.015	0.240 ± 0.011	12.16 ± 0.43
ZnCl_2	0.5	10	0.00250 ± 0.0005	0.165 ± 0.008	6.97 ± 0.32
$\text{Zn(NO}_3)_2$	50	10	0.0011 ± 0.00016	0.185 ± 0.009	8.11 ± 0.46
$\text{Cd(NO}_3)_2$	0.5	4	0.0052 ± 0.0009	0.274 ± 0.012	15.61 ± 1.34
$\text{Pb(NO}_3)_2$	0.5	4	0.0025 ± 0.0008	0.106 ± 0.005	6.54 ± 0.30
$\text{Cu(NO}_3)_2$	0.5	3	0.0023 ± 0.0003	0.092 ± 0.004	5.80 ± 0.26
$\text{Ni(NO}_3)_2$	0.5	4	0.0008 ± 0.0002	0.049 ± 0.004	5.23 ± 0.24

Table 6

Influence of pH on the partition coefficient. a) membrane/aqueous phase partition coefficient. The pH of the soak and receiver solutions were the same. The pH was adjusted in b) with HCl and in c) with HNO_3 .

Salt	pH	Partition coefficient	a)
CaCl_3	6.2	0.3	b)
"-	4.7	0.9	b)
MgCl_2	5.8	0.04	b)
"-	4.7	0.08	b)
ZnCl_2	4.7	0.0030	b)
"-	2.1	0.0025	b)
$\text{Zn}(\text{NO}_3)_2$	4.7	0.0010	c)
"-	3.7	0.0006	c)

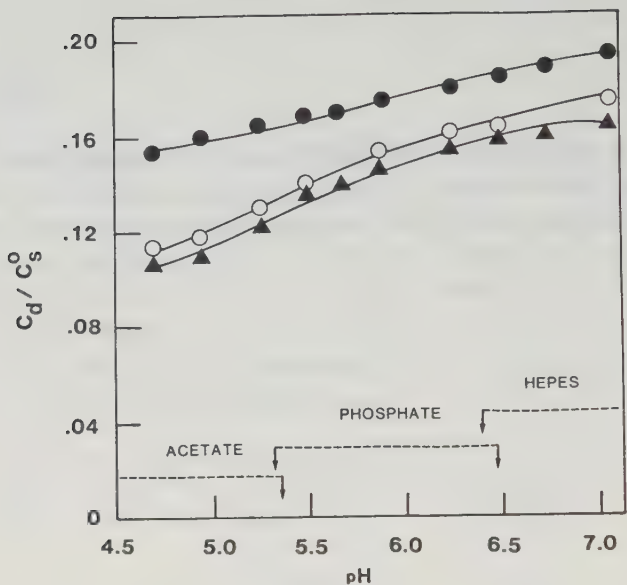


Fig 1. Influence of the pH of the sample on the dialysis of zinc ions. Flow dialysis; flow rate 0.5 ml min^{-1} . Sample and receiver solutions with identical composition. Receiver solution: ● pH 4.7; ○ pH 6.5; ▲ pH 6.9.

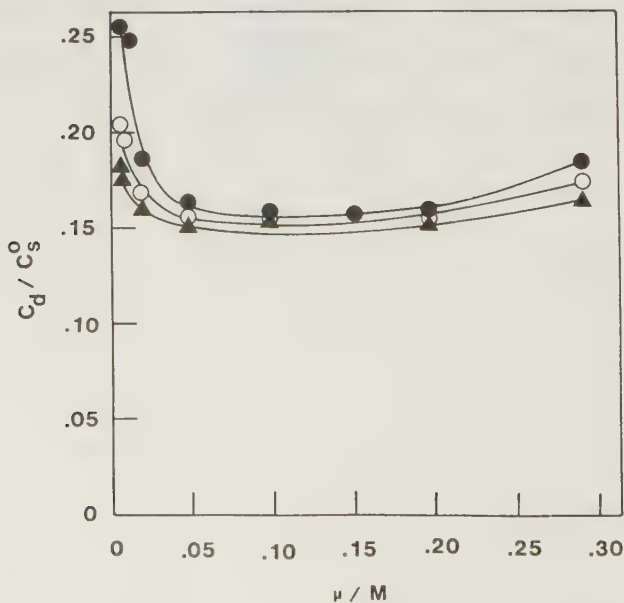


Fig. 2. Influence of ionic strength on the dialysis of zinc ions. Flow dialysis; flow rate 0.5 ml min^{-1} . Sample and receiver solutions at the same pH and ionic strength. ● pH 4.7; ○ pH 5.5; ▲ pH 6.5.

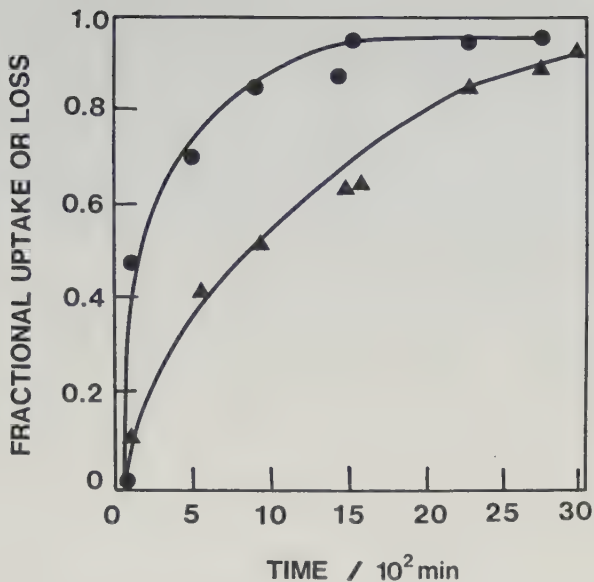


Fig. 3. Sorption and desorption of zinc ions from a package of ten cellulose acetate membranes. Soak solution: ZnCl_2 , acetate buffer, pH 5.5, $u = 0.15$ M. Receiving solution: acetate buffer, pH 5.5, $u = 0.15$ M. ● sorption ▲ desorption

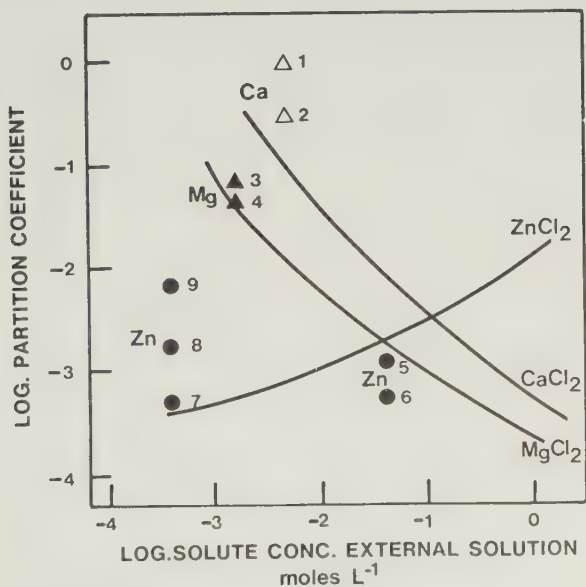


Fig. 4. Partition coefficients of Zn(II) , Mg(II) and Ca(II) salts. 1. CaCl_2 ; 2. CaCl_2 + histidine; 3. MgCl_2 ; 4. MgCl_2 + histidine; 5. $\text{Zn(NO}_3)_2$; 6,7. ZnCl_2 ; 8. ZnCl_2 + histidine; 9. ZnCl_2 + CaCl_2 + histidine.

Eduardo O. Martins

Department of Analytical Chemistry, University of Lund

P.O.Box 124, S-221 00 Lund, Sweden

SUMMARY

Dialysis of zinc(II) through cellulose-acetate membranes was studied in a flow-through dialyzer. The metal was determined with differential pulse anodic stripping voltammetry in a flow-through cell with a detection limit of 10^{-9} M zinc(II). The concentration of zinc(II) in the dialysate decreased by 750 times when albumin was added in an excess corresponding to the physiological concentrations. Addition of amino acids in physiological concentrations, had little or no effect on the transfer of zinc(II) through the membrane. Albumin solutions and a model serum containing inorganic salts, amino acids, lipids and carbohydrates were compared with pooled serum. Zinc(II) was transferred faster from the latter at equal concentrations of exchangeable total zinc. Predialysis of the pooled serum decreased the zinc(II) transferring capability indicating some unknown mode of transport from the untreated serum.

A deeper understanding of the zinc metabolism in the body requires knowledge about the distribution of the metal on various types of ligands and in different pools. Many of the non-exchangeable pools can be assessed individually, e.g. by determination of an enzyme or another macromolecule. Other pools are of secondary interest because of the extremely slow mobilization, e.g. the bones or the zinc complexes in the brain or in the erythrocytes. The zinc(II) content of the exchangeable pools in the serum is a prime target for mechanistic studies because changes (mostly decreases) have been observed in a great number of pathological conditions.

Methods which can give information about the various exchangeable zinc(II)-species should be of great value as complement to methods, e.g. atomic absorption, which measures the total amount of metal. Dialysis is one of the few methods which can give an overall picture of zinc(II) bound to small ligands. Equilibrium dialysis is very time-consuming and a flow-through method was therefore developed. The determination was made by stripping voltammetry which excludes non-ionic zinc compounds. The low zinc(II)-levels in the dialysates make it necessary to select a procedure with a very low detection limit.

EXPERIMENTAL

Apparatus

A flow-through dialyzer (Tecator AB, Höganäs) with 500 mm long rectangular channels, height 0.091 mm and width 1.5 mm, was used throughout. It was made from two machined Perspex halves which was clamped together with a cellulose acetate dialysis membrane (Elkey Products Inc., Worcester, Mass. Cat. No. DM-C-2-157-0144) in between. The dialyzer and some of its properties have been described before [1,2].

The dialyzer was incorporated into a manifold, see Fig. 1, in which a sample, 2.0 ml, was introduced by a pneumatic valve into a carrier stream consisting of 0.15 M NaCl + 10 mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane) buffer, pH 7.4. The dialyzed components were

received by a stream of the same buffer pumped with the same flow rate, 0.5 ml min^{-1} , as the sample carrier stream. The receiver channel was mixed with a pH 5.5 buffer, $0.15 \text{ M NaCl} + 50 \text{ mM sodium acetate}$, 0.5 ml min^{-1} . The detector was a flow-through voltammetric cell with a hanging mercury drop electrode.

Zinc(II) was plated into the mercury while the zone containing the dialyzed components passed the cell. The metal was then determined by differential pulse anodic stripping with a sweep from the deposition potential, -1.2 V vs SCE , to -850 mV . The cell and procedure has been described earlier [3].

The zinc(II) response was unchanged when cholesterol or fatty acids were added to the standard solutions in amounts corresponding to those expected to pass the membrane in dialysis of a synthetic serum sample.

The solutions were degassed pumped with a peristaltic pump (Gilson, Minipuls 2) with a gas-tight box around the pumphead. The injector and the dialyzer were also mounted in the box. Teflon tubings outside the box were surrounded coaxially by another tubing made of nylon. The annuli as well as the box around the pumphead were flushed with oxygen-free ($<0.01\%$) nitrogen. Another valve was used for rinsing the flow system with water.

The total time needed for a zone to pass the detector cell was four minutes and another minute was needed for the voltammetric sweep. The wash time was also one minute so that the total capacity became $10 \text{ samples h}^{-1}$.

Trace metal purification

Columns, 300 ul , containing 8-quinolinol immobilized to porous glass (Pierce Chemical Co) were inserted in the pump lines for the buffers to remove trace metals on-line [4]. The sample solutions were prepared from buffers which had been purified electrolytically over a stirred mercury pool kept at -1.5 V vs SCE for 72 hours. All glassware were acid-washed with 2 M HNO_3 for at least 48 hours and

rinsed in water, which had been purified in a Millipore-Q unit. The other chemicals were used as received, the inorganic salts were of pro analysi quality (Merck AG) and the organic chemicals were from Sigma Chemicals Co. The serum albumin was fraction V, fatty acid free. Cholesterol and the cholesterol esters were purified by HPLC.

RESULTS

Solutions with different initial concentrations of zinc(II) were titrated with serum albumin while fractions were analyzed by on-line dialysis and zinc determinations, see Fig. 2. Zinc(II) forms a strong 1:1 complex with albumin, binding constant = $10^{6.9} \text{ M}^{-1}$ as well as weaker complexes with more than one zinc(II) per albumin molecule [5-7]. The amount of free zinc(II) decreases as more albumin is added and this is reflected by a decrease of the zinc(II) concentration in the dialysate. The titration was continued until the solution composition corresponded to that of serum (the large circle in the insert in Fig. 2). At this point the dialyzable zinc(II) fraction was about 750 times less than in a solution with only zinc(II) and no albumin. The behaviour can also be described by saying that the dialysis factor for zinc(II) decreased from 0.160 in albumin free solutions to 0.00021 in 0.6 mM albumin. The dialysis factor is defined as the output concentration in the detector channel divided by the input concentration in the sample channel.

Amino acids form zinc(II) complexes which dialyze through the membrane with about 80% of the rate of zinc(II) [8]. The effect was studied by adding albumin to solutions, which besides zinc(II), also contained a mixture of 19 amino acids, each one in a concentration corresponding to that in serum, (Table 1). The titrations (Fig. 3) show that the transport of zinc(II) increases substantially when the zinc(II) and albumin are present in about equimolar quantities. The solubilizing effect of amino acids decreases, however, as the albumin excess increases, and becomes insignificant at physiologically relevant concentrations.

The effect was also studied by titrating a zinc(II)-albumin solution with the individual amino acids, one at a time. Increased dialysis was observed only with histidine, methionine and glycine in excess, see Fig. 4. The right part of the figure is physiologically unrealistic except e.g. in histidinemia. The results indicate that the presence of amino acids should have little effect on the dialysis of zinc(II) from a serum sample through a cellulose acetate membrane.

The zinc(II) binding of albumin, albumin + amino acids, a synthetic serum with the composition given in Table 1, pooled serum and dialyzed serum were compared. Standard additions of zinc(II) was made to the solutions and the zinc concentrations in the dialysate was measured, see Fig. 5. It can be seen that all the solutions bind the zinc(II) very effectively (the insert in Fig. 5) in non-dialyzable form at physiological concentrations. The dialysis of zinc(II) increases rapidly as more metal ions are added and large differences between the solutions become apparent. The synthetic serum seems to bind zinc(II) stronger than any of the other solutions at a level of 100-200 μM zinc(II). Separate experiments showed that the effect was associated with addition of the lipid fraction. As more zinc(II) is added the zinc(II) binding becomes about the same as that of the albumin solutions.

The dialysis of zinc(II) from a serum sample becomes increasingly larger than from a synthetic solution as the total zinc(II) concentration increases. This indicates either that some unidentified components of serum interact with the albumin so that the fraction of free zinc ions increases or that some dialyzable serum components are able to compete with the albumin in binding the zinc(II). A serum which had been predialyzed for 24 hours shows a behaviour which comes close to that of the synthetic serum. This indicates that the molecules which are responsible for the enhanced dialysis of zinc(II) have been removed to a large extent during the predialysis.

DISCUSSION

The rate of mass transfer between the channels in flow-through non-equilibrium dialysis is determined jointly by the membrane

permeability and by the mass transfer in the aqueous solutions. The latter dominates by a factor of four to five for zinc(II) in the present dialyzer at 0.5 ml min^{-1} [1]. The diffusion coefficient for albumin [9] and probably also for the zinc-albumin complex is 100 times smaller than that of zinc(II).

In a layer close to the membrane several steps will occur simultaneously: free zinc will pass the membrane into the detector channel, the zinc-albumin complex will dissociate, free zinc(II) and zinc-albumin complex will move in from layers further away and albumin will move out from the membrane. The combined effect cannot be predicted quantitatively until a mathematical model has been derived which takes all these processes into account. Non-linear concentration dependence has been observed with dissociating complexes [10] but is not expected for zinc(II) in solutions of physiological composition. The linearity in the latter case was verified experimentally, although only with moderate precision.

The results obtained with equilibrium dialysis will not depend on the transport parameters once the equilibrium has been reached. If an unknown dialyzable ligand is present in serum, as indicated by the results in Fig. 5, the interpretation may again be far from straightforward. One study [11] reports on the equilibrium dialysis of zinc(II) from albumin solutions. No direct comparisons can be made, however, because of the large differences in the zinc(II)/albumin ratio.

Serum samples were ultrafiltered by application of pressure and zinc was determined in the filtrate [12]. The concentration of zinc(II) in the filtrate was of the same size as the concentration of dialyzable zinc(II) reported in this work. Extensive dialysis against renewed metal-free solutions has been used to remove all exchangeable zinc(II) prior to determinations of the metal in the remaining solution [11]. Zinc(II) bound to macromolecules has been determined with chromatography or electrophoresis [13-16]. These methods will determine the concentration of the individual zinc(II)-carrying stable ligand. There is thus a fundamental difference between these methods and the dialysis and ultrafiltration which measure the

concentration of zinc(II) bound to all small dialyzable ligands. Only analysis of samples from individuals with various diagnosed disturbances can give information about the relative merits of two approaches.

Inspection of the titration curves in Fig. 2 and 3 shows that the very large excess of albumin in physiological solutions is of the greatest importance for retaining zinc(II) in the serum. The amino acids in serum did not increase the rate of dialysis of zinc(II) under these conditions. Neither did the other constituents of the synthetic serum, including the tri- and the tetrapeptides, on the contrary, the transfer rate of zinc(II) was lower than in the albumin solution. The transport of zinc in the body is through the blood, which contains about 1.3% of the total body pool. Nevertheless a high zinc-binding strength might be of importance to reduce zinc losses. The medical literature seems to indicate that the major problems are associated with loss of zinc through the urine with a resulting decrease of the total zinc of the serum.

The observation (Fig. 5) that the pooled serum leaks zinc(II) through a dialysis membrane about twice as fast as the serum albumin solution, and three times as fast as the synthetic serum is very interesting. The reduction of leakage after predialysis of the serum indicates another zinc(II)-solubilizing mechanism than those considered previously. There is, however, very great differences between the transport properties of a cellulose acetate membrane and a biological membrane and the observation may not have any clinical significance.

ACKNOWLEDGEMENTS

The author expresses his gratitude to Professor Gillis Johansson for valuable discussions and help with the manuscript. This work was supported by grants from the Swedish Natural Science Research Council.

REFERENCES

1. B. Bernhardsson, E. Martins and G. Johansson, *Anal. Chim. Acta*, in press.
2. E. Martins, M. Bengtsson and G. Johansson, *Anal. Chim. Acta*, in press.
3. E. Martins and G. Johansson, *Anal. Chim. Acta* 140(1982)29.
4. F. Malamas, M. Bengtsson and G. Johansson, *Anal. Chim. Acta* 160(1984)1.
5. R. Österberg, *Acta Chem. Scand.* 25(1971)3827.
6. E.L. Giroux and R.I. Henkin, *Biochim. Biophys. Acta* 273(1972)64.
7. E.O. Martins and T. Drakenberg, *Inorg. Chim. Acta* 67(1982)71.
8. E. Martins and G. Johansson, *Anal. Chim. Acta* 116(1980)53.
9. R.F. Probststein, W.F. Leung and Y. Alliance, *J. Phys. Chem.* 83(1979)1228.
10. L.C. Craig, T.P. King and A. Stracher, *J. Am. Chem. Soc.* 79(1957)3729.
11. A.S. Prasad and D. Oberleas, *J. Lab. Clin. Med.* 76(1970)416.
12. R.C. Whitehouse, A.S. Prasad and Z. T. Cossack, *Clin. Chem.* 29(1983)1974.
13. J.W. Foote and H.T. Delves, *Analyst*, 108(1983)492.
14. P.E. Gardiner, J.M. Ottaway, G.S. Fell and R.R. Burns, *Anal. Chim. Acta* 124(1981)281.
15. G.W. Evans and T.W. Winter, *Bioch. Biophys. Res. Comm.* 66(1975)1218.
16. J.B. Dawson, M.H. Bahereyni-Toosi, D.J. Ellis and A. Hodgkinson, *Analyst* 106(1981)153.

Table 1

	synthetic serum		human serum normal values	
HCO_3^-	23-30	mmoles L^{-1}	21-25	mmoles L^{-1}
HPO_4^{2-}	1.5	"	0.8-1.6	"
Cl^-	100-150	"	94-111	"
K^+	3-8	"	36-54	"
Na^+	135-150	"	137-147	"
Ca^{2+}	2-2.5	"	2.3-2.7	"
Mg^{2+}	1-1.5	"	0.8-1.0	"
Cu^{2+}	0-50	$\mu\text{moles L}^{-1}$	11-24	$\mu\text{moles L}^{-1}$
GLY	212-675	$\mu\text{moles L}^{-1}$	237	$\mu\text{moles L}^{-1}$
HIS	61-91	"	74	"
CYS	25-118	"	10-33	"
ASP	22-78	"	44	"
THR	53-135	"	129	"
TYR	50-135	"	52	"
GLU	237-654	"	568	"
PHE	25-76	"	33	"
SER	30-150	"	115	"
MET	25-72	"	23	"
TRY	27-55	"	48	"
LEU	85-138	"	111	"
ISO	17-65	"	63	"
ALA	142-382	"	336	"
ASPac	10	"	8	"
GLUac	63	"	58	"
ARG	68-75	"	75	"
LYS	44-155	"	153	"
VAL	158-237	"	214	"
HIS-GLY-GLY	~ 25	$\mu\text{moles L}^{-1}$		
GLY-HIS-GLY-GLY	~ 30	"		
Palmitic ac	1.36	mmoles L^{-1}		
Stearic ac	0.96	"		
Triglycerol	~ 0.6	"	0.23-1.8	mmoles L^{-1} (triglycerids)
Glyceryl triacetate	0.23-1.7	"	4.5-1.0 g/L	(total lipids)
β -glycerolphosphate	0.15-0.53	"		
Cholesterol	2.1-8.0	"	up to 6.5	mmoles L^{-1}
Cholesterol-esters	~ 750	$\mu\text{moles L}^{-1}$		
D(+)-glucosamine	100-579	"		
L-glutamine	78-385	"		
Glucose	1.5-6.0	mmoles L^{-1}	2.8-5.6	mmoles L^{-1}
Heparin	100 USP units ml^{-1}			
Albumin	0.55-0.65	mmoles L^{-1}	0.6	mmoles L^{-1}
Transferrin	2.5-5.0	$\mu\text{moles L}^{-1}$	2.85-4.3	$\mu\text{moles L}^{-1}$
Bilirubin	0-30	$\mu\text{moles L}^{-1}$	up to 17	$\mu\text{moles L}^{-1}$ (total)
Urea	2.5-8	mmoles L^{-1}	3.3-7.5	mmoles L^{-1}
Creatine	50-100	$\mu\text{moles L}^{-1}$	70-115	$\mu\text{moles L}^{-1}$
Creatinine	50-120	$\mu\text{moles L}^{-1}$	53-97	$\mu\text{moles L}^{-1}$

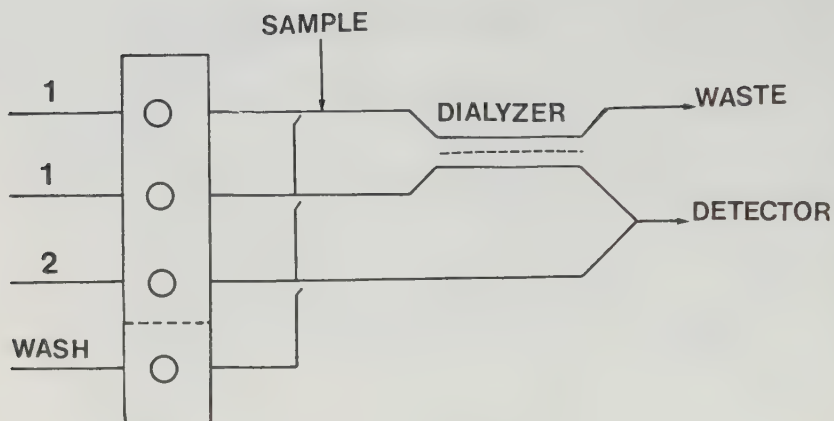


Fig. 1. Schematic diagram of the flow-through dialysis system

1. 0.15 M NaCl + 10 mM HEPES, pH 7.4
2. 0.15 M NaCl + 50 mM Acetate buffer, pH 5.5

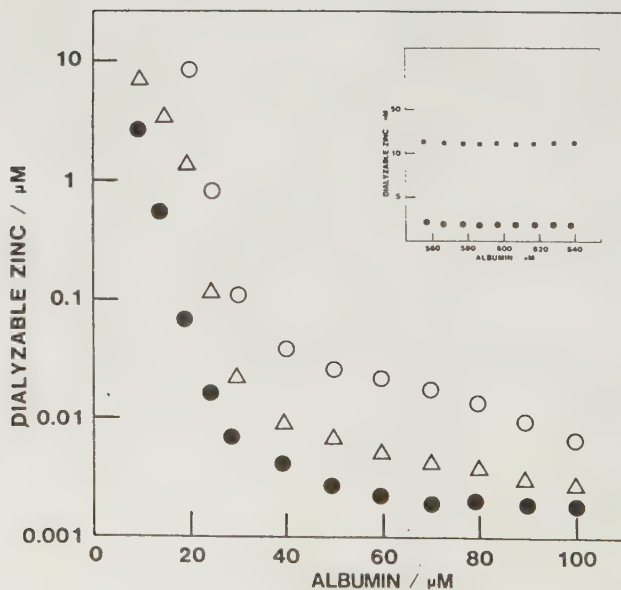


Fig. 2. Titration of zinc with serum albumin, $u = 0.15$ M, pH 6.9

Zinc concentration: ● 15 μ M Δ 38 ○ 76 ■ 311

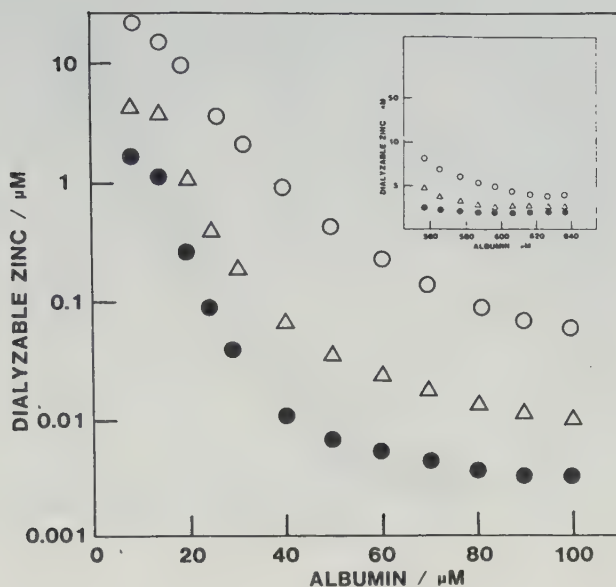


Fig. 3. Titration of zinc-amino acids with serum albumin.
Concentration of amino acids as in Table I (mid-range values). Zinc concentration: ● 15 μM △ 38 ○ 76

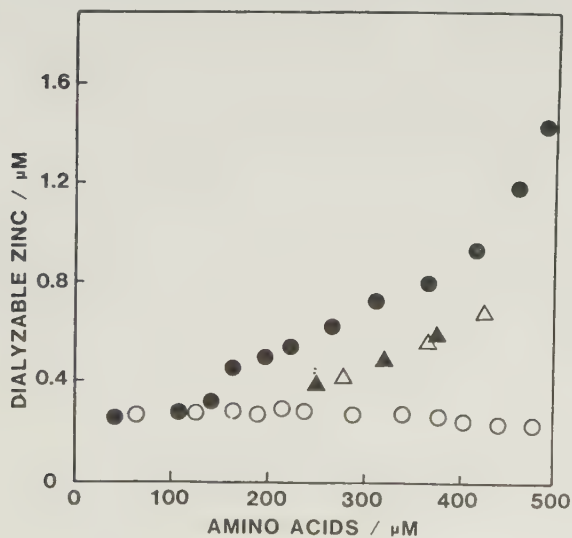


Fig. 4. Titration of zinc-albumin-amino acids with amino acids.
Concentration of amino acids in the solution as in Table I (mid-range values). Zinc, 15 μM , BSA 0.6 mM, $u = 15$, pH 7.4
● histidine ▲ glycine △ methionine ○ other

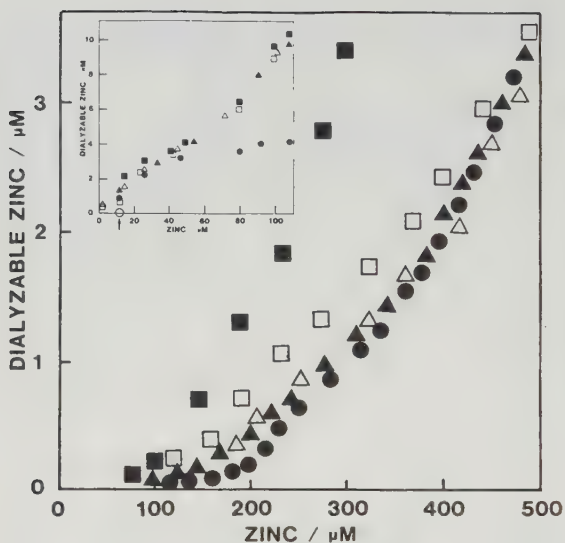


Fig. 5. Titration of synthetic and human sera with zinc

■ human serum □ pre-dialyzed human serum ▲ serum
 albumin + amino acids △ pre-dialyzed serum albumin
 ● serum albumin + fatty acids + amino acids

